CONF-790951--1

Invited Paper:
In Vitro Toxicity for
Environmental Agents,
NATO Ad. Res. Inst.

QUANTITATIVE MAMMALIAN CELL MUTAGENESIS AND MUTAGEN SCREENING: STUDY WITH CHO CELLS[1]

Abraham W. Hsie, J. Patrick O'Neill, Juan R. San Sebastian[2] and Patricia A. Brimer

Biology Division, Oak Ridge National Laboratory and The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee U.S.A.

[1]Research supported jointly by the Environmental Protection Agency (IAG-D8-E681), the National Center for Toxicological Research, and the Office of Health and Environmental Research, U. S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation. JRSS is a Monsanto Fellow.

[2]Postdoctoral Fellow of Monsanto Fund.

Send Proofs to: Abraham W. Hsie, Ph.D.

Biology Division

Oak Ridge National Laboratory

P. O. Box Y

Oak Ridge, TN 37830

FOOTNOTE

Abbrevations: TG, 6-thioguanine; TG^r, TG-resistant or TG-resistance; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; CHO cells, Chinese hamster ovary cells; EMS, ethyl methanesulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; iPMS, isopropyl methanesulfonate; DES, diethylsulfate; ENNG, N-ethyl-N'-nitro-N-nitrosoguanidine; B(a)P, benzo(a)pyrene; 4-NQO, 4-nitroguinoline 1-oxide; DMS, dimethylsulfate; MMS, methyl methanesulfonate; MNU, N-methyl-N-nitrosourea; ENU, N-ethyl-N-nitrosourea; BNU, N-butyl-N-nitrosourea; DMN, dimethylnitrosamine.

Advances in cell biology within the last two decades enable the use of mammalian cells in culture for mutation research. A simple, sensitive and quantitative mammalian cell specific locus mutational assay would be useful for studying mechanisms of mammalian mutation, and for assessing the mutagenic potential of environmental agents to humans.

Several mammalian cell mutation systems (3), especially those utilizing resistance to purine analogues such as 8-azaguanine and 6-thioguanine (TG)[1] as a genetic marker, have been developed. The selection for mutation induction to purine analogue resistance is based on the fact that the wild-type cells containing hypoxanthine-guanine phosphoribosyl transferase (HGPRT) activity are capable of converting the analogue to toxic metabolites, leading to cell death; the presumptive mutants, by virtue of loss of HGPRT activity, are incapable of catalyzing this detrimental metabolism and, hence, escape the lethal effect of the purine analogue.

We have chosen to use a near-diploid Chinese hamster ovary (CHO) cell line for our mutagenesis study because they are perhaps the best characterized mammalian cells genetically. They exhibit nearly 100% cloning efficiency under normal growth conditions, and are capable of growing in a relatively well-defined medium with a short population doubling time of 12 to 14 hr. In addition, the cells have a stable, easily recognizable karyotype of 20 or 21 chromosomes (depending on the subclone) and are thus suitable for studies of mutagen-induced chromosome and chromatid aberrations, and sister-chromatid exchange. Karyotypic stability ensures the functionally monosomic state of the HGPRT gene localized in the X chromosome of CHO cells.

In this article, we summarize the development of the CHO/HGPRT system for quantifying mutation to TG resistance, evidence of the genetic basis of

of mutation at the HGPRT locus, the quantitative and sensitive nature of the assay, interrelationships of cellular lethality and mutation as affected by various mutagens, quantitative analyses of exposure dose and the relationship between mutation induction and DNA lesion, structure-activity (mutagenicity) of various classes of direct-acting mutagens and promutagens, the use of this assay in mutagen screening, correlation between animal carcinogenicity with CHO mutagenicity, and the amplication of the CHO/HGPRT assay to a Multiplex Genetic Toxicology System.

MATERIALS AND METHODS

Cell Culture

All studies to be described have employed a subclone of CHO-K_1 cells designed as CHO-K_1-BH_4 (10). Cells are routinely cultured in Ham's F12 medium (K. C. Biological Co.) containing 5% heat-inactivated (56°C, 30 min), extensively dialyzed fetal calf serum (medium F12FCM5) in plastic tissue culture dishes (Falcon or Corning Glass Works) under standard conditions of 5% CO_2 in air at 37°C in a 100% humidified incubator. The population doubling time is 12 to 14 hr.

Treatment with Chemicals

Cells are plated at 5 X 10^5 cells/25 cm^2 bottle in medium F12FCM5. After a 16- to 24-hr growth period (cell number = ~1.0 to 1.5 x 10^6 cells/plate), the cells are washed twice with saline G, and sufficient serum-free F12 medium is added to bring the final volume to 5 ml after the addition of various amounts of microsome preparation (up to 1 ml) and 50 µl of mutagen, usually dissolved in dimethyl sulfoxide. Mutagens and/or microsomes are omitted from some plates to provide controls. The microsomal preparation is

prepared from livers of Aroclor 1254-induced male Sprague-Dawley rats; the microsome mix for biotransformation contains (per ml) 30 µmoles KCl, 10 µmoles $MgCl_2$, 10 µmoles $CaCl_2$, 4 µmoles NADP, 5 µmoles glucose-6-phosphate, 50 µmoles phosphate buffer (pH 8.0), and 0.1 ml microsome fraction (which contains 3 to 4 mg protein). Cells are then incubated for 5 hr and washed 3 times with saline G before 5 ml of F12FCM5 is added. After they are incubated overnight, cells are dissociated with 0.05% trypsin, and plated for cytotoxicity and specific gene mutagenesis to be described below (10, 22).

Cytotoxicity

For an expected cloning efficiency higher than 50%, 200 well-dispersed single cells are plated, and for an expected survival lower than this, the number of cells plated is adjusted accordingly to yield 100 to 200 surviving in medium F12FCM5 for 7 days. Control cells, which do not receive treatment with mutagen, usually give 80% or higher plating efficiency. Neither the solvent-microsome mix nor these agents individually affects the cellular cloning efficiency. The effect of a mutagen on the cloning efficiency is expressed as percent survival relative to the untreated controls (10, 22).

Specific Gene Mutagenesis

For the determination of mutation induction, the treated cells are allowed to express the mutant phenotype in F12 medium for 7 to 9 days, at which time mutation induction reaches a maximum. Routine subculture is performed at 2-day intervals during the expression period, and at the end of this time the cells are plated for selection in hypoxanthine-free F12FCM5 containing 1.7 µg/ml (10 uM) of TG at a density of 2.0×10^5 cells/100-mm plastic dish (Corning or Falcon), which permits 100% mutant recovery.

After 7 to 8 days in the selective medium, the drug-resistant colonies develop; they are then fixed, stained, and counted. Mutation frequency is calculated based on the number of drug-resistant colonies per survivor at the end of the expression period (10, 22).

RESULTS AND DISCUSSION

Development of a Protocol for Quantifying Specific Gene Mutagenesis

Various mutants with phenotype(s) different from the parental mammalian cells have been isolated after mutagen treatment. Since the purpose of most studies was to obtain a particular type of phenotypic variant for genetic, biochemical, or molecular analysis, the procedure for mutation induction generally does not take into consideration of the quantitative aspects of the mutagenesis. Due to the intrinsic characteristics of the CHO/HGPRT assay, factors required to quantify mutagenesis need to be established individually. These include:

Mutagen treatment and the physiological state of cell growth: Except in those experiments designed for cell-cycle study, we treat cells during the exponential growth state because some mutagens may act preferentially on the proliferating cells.

Medium for cell growth and mutant selection: The growth medium used should not allow preferential growth of either wild type or mutants. This is especially crucial for such mutants as TG^r which require long phenotypic expression time (10,22). A slight advantage or disadvantage for mutant growth will grossly distort the observed mutation frequency. During mutant selection with TG, the selective medium is devoid of hypoxanthine because TG competitively

inhibits hypoxanthine transport across cell membrane (22) and the reverse is likely true.

TG concentration: Optimum TG concentration should be used to select for phenotypic variants of mutational origin which are neither leaky nor epigenetic in nature (22).

Cell density for selection: For full mutant recovery, the ratio of mutants to wild-type cells should be such that the mutants will not be affected by the lethal effects of the purine analogue metabolites cross fed by the wild-type cells (22).

Phenotypic expression time: Since the selection of the mutants is based on the loss of the HGPRT activity, a period of delay for expressing the TG^r phenotype is expected to allow completion of mutation fixation and dilution of the preexisting enzyme and mRNA coded for HGPRT. We have found that maximum stable expression of the TG^r phenotype is reached 7 to 9 days after mutagenesis and remains constant thereafter irrespective of the nature and dose of the mutagen (9, 21-23, 27).

Characteristics of the CHO/HGPRT System: Evidence of the Genetic Basis of at a Specific Locus

Since the state of the art of somatic cell genetics remain short of providing a direct proof that the altered phenotype(s) observed has resulted in a modified nucleotide sequence of the specific gene, causing modified coding properties which result in the production of altered protein with changes in the amino acid sequence, we, thus, relied on establishing indirect criteria which are consistent with the concept that the observed phenotypic variants are genetic in nature (3, 32, 36, 39).

Over the past 5 years, we have used this assay protocol and have found in over 500 experiments that the spontaneous mutation frequency lies in the range of 0 to 10 x 10^{-6} mutant/cell. A detailed fluctuation analysis of spontaneous mutation showed that the spontaneous mutant phenotype arises in a random manner, as would be expected for a mutational event. The mean values for the mutation rate are 7.0 x 10^{-8} (F_o method) and 32.6 x 10^{-8} (mean method) mutation/cell/generation (26). Various physical and chemical agents are capable of inducing TG resistance. In all chemical mutagens examined, mutation induction occurs as a linear function of the concentration (4-7, 9, 10, 13-17, 21-28, 38). For example, mutation frequency increases approximately linearly with ethyl methanesulfonate (EMS) concentration in the near-diploid CHO cell line, conforming to the expectation that mutation induction occurs in the gene localized at the functionally monosomic X chromosome. However, in the tetraploid CHO cells, EMS does not induce an appreciable number of mutations, even at very high concentrations, as predicted theoretically (13).

We found a very low ($<10^{-7}$) spontaneous reversion with 13 TG^r mutants, all of which contain low, yet detectable, HGPRT activity. More than 98% of the presumptive mutants isolated either from spontaneous mutation or as a result of mutation induction are sensitive to aminopterin, incorporate hypoxanthine at reduced rates, and have less than 5% HGPRT activity (13, 22). Studies in progress have also shown that a high proportion of TG^r mutants containing temperature-sensitive HGPRT activity suggesting that mutation resides in the HGPRT structural gene (J. P. O'Neill and A. W. Hsie, unpublished observations).

The CHO/HGPRT system appears to fulfill the criteria for a specific-gene-locus mutational assay.

Interrelationships of Mutagen-Mediated Cellular Lethality and Mutation Induction

When EMS was employed as a mutagen, mutation induction occurred over the entire survival curve ("EMS" type) (10, 13, 21) including a shoulder region, where there is no appreciable loss of cell survival. Apparently there is no threshold effect of mutation induction with EMS. X rays (23), UV light (11), ICR-191 (24), isopropyl methanesulfonate (iPMS) (7), and diethylsulfate (DES)(8) also exhibit "EMS-type" curves. However, there are agents, typified by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (6, 22) and N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) (6) which do not exhibit appreciable shoulder regions in the survival curve, and for which mutation induction always occurs concomitantly with the loss of cell survival ("MNNG type").

Most promutagenic agents are neither toxic nor mutagenic to the cells in the absence of S_9-mediated metabolic activation. With S_9, benzo(a)pyrene [B(a)P] is mutagenic and cytotoxic, displaying an "EMS-type" curve (P. A. Brimer, J. P. O'Neill, J. R. San Sebastian, and A. W. Hsie, unpublished). The direct-acting mutagen 4-nitro-quinoline-1-oxide is highly cytotoxic and mutagenic to the CHO cells, and its cytotoxicity and mutagenicity decrease when it is treated with S_9 (J. R. San Sebastian and A. W. Hsie, unpublished). Thus, cytotoxicity and mutagenicity of chemical mutagens are separable.

Quantitative Analysis of Chemical-Induced Cellular Lethality and Mutagenesis

A study of EMS exposure dose: Earlier, we found that EMS-induced mutation frequency to TG^r in cells treated for a fixed period of 16 hr is a linear function over a large range of mutagen concentrations. Further studies with varying concentrations (0.05 to 3.2 mg/ml) of EMS for 2 to 12 hr showed that

the manifestation of cellular lethality and mutagenesis occurs as a function of EMS exposure dose; i.e., these two biological effects are the same for different combinations of concentration multiplied by duration of treatment which yield the same product. From these studies the mutagenic potential of EMS can be described as 310×10^{-6} mutant (cell mg ml^{-1} $hr)^{-1}$ (21). Thus, the CHO/HGPRT system appears to be suitable for dosimetry studies.

Relationship between DNA alkylation and mutation induction to TG^r by N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU): We treated cells with MNU or ENU, and found that both alkylation and mutation induced by MNU and ENU increases linearly with increasing concentrations. At equimolar concentrations, MNU has 15 times the DNA alkylating activity of ENU, but only 3 times the mutagenic activity. Thus, in terms of mutation induction per unit alkylation, ethylation of DNA by ENU appears to result in a 5-fold greater fraction of mutagenic lesions than does methylation by MNU. Ethylation appears to result in a higher proportion of mutagenic lesions than does methylation. This may reflect either a higher frequency of miscoding events such as O-6-guanine alkylation or an effect of ethylation per se (38).

Mutagenicity, cytotoxicity, and DNA-binding of Pt(II)chloroammines: We have studied the mutagenicity of Pt(II)chloroammines. Based on the slope of the linear dose-response curve, the mutagenicities [expressed as mutants/10^6 cells per μM of Pt(II)chloroammine] are: cis-$Pt(NH_3)_2Cl_2$, 47; trans-$Pt(NH_3)_2Cl_2$, 0.4; K_2PtCl_4, 0.7; $[Pt(NH_3)_4]Cl_2$, 0.01. Cytotoxicities follow the same relative order and are of similar magnitude. By use of radioactive ^{195m}Pt, we observed that after incubation for 16 hr, cis-$Pt(NH_3)_2Cl_2$ binds linearly over a concentration range of 0.4 to 1.7 μM; the slope of the curve is 7.9×10^{-11} µmol Pt bound per nucleotide per μM of cis-$Pt(NH_3)_2Cl_2$ added.

Preliminary results indicate that trans-$Pt(NH_3)_2Cl_2$ binds to DNA to a similar extent. It appears that the chemical nature of the lesions produced in the DNA rather than the number of molecules bound to DNA is responsible for the different biological potencies of these Pt compounds (19).

Structure-activity relationship of direct-acting chemical mutagens

Alkylating chemicals (total of 11): The dose-response relationships of cell killing and mutation induction of two alkylsulfates [dimethylsulfate (DMS) and DES] and three alkyl alkanesulfonates [methyl methanesulfonate (MMS), EMS, and iPMS] have been compared under identical experimental conditions. Based on mutants induced per unit mutagen concentration, both the cytotoxicity and mutagenicity decreased with the size of the alkyl group: DMS > DES; MMS > EMS > iPMS (7).

Similar comparative studies were extended to two nitrosamidines (MNNG and ENNG) and three nitrosamides [MNU, ENU, and N-butyl-N-nitrosourea (BNU)]. The order of their relative mutagenic activity was MNNG > ENNG > MNU > ENU > BNU (6).

All of these 10 alkylating agents are known to be carcinogenic, and are mutagenic in our assay. Nitrosation appears to be essential for mutagenicity of nitrosoamidines; N-methyl-N'-nitroguanidine (MNG), a non-carcinogenic analogue of MNNG, is not mutagenic even at concentrations 50,000 higher than its nitroso analogue, MNNG (J. R. San Sebastian and A. W. Hsie, unpublished).

Heterocyclic nitrogen mustard (ICR compounds) (total of 19): We have studied the cytotoxicity and mutagenicity of 19 ICR compounds (ICR 449, 217, 220, 191, 191-OH, 170, 170-OH, 283, 171, 372, 372-OH, 340, 340-OH, 342, 371, 355, 292, 292-OH, 368). Thirteen of these compounds are mutagenic. At

equimolar concentrations, the compounds with the tertiary-amine-type side chain (ICR 217, 340, 355, 368, 170, and 292) are more mutagenic than the compounds with the secondary-amine-type side chain (ICR 449, 371, 191, and 372). All secondary-amine types show a "plateau" in their concentration-dependent mutagenesis curves at 3 to 4 μM. Shortening of the side chain by one carbon (ICR 171) results in a reduced mutagenicity. Substitution of a sulfur atom for a nitrogen in the side chain (ICR 342) increases both mutagenicity and cytotoxicity. The presence of two 2-chloroethyl groups on the side chain (ICR 220) also results in greatly increased cytotoxicity and mutagenicity. When the 2-chloroethyl group of ICR 340, 372, 292, 191, or 170 is replaced by a 2-hydroxyethyl group (ICR 340-OH, 372-OH, 292-OH, 191-OH, 170-OH), a mutagenically inactive compound results which remains toxic; this indicates that the 2-chloroethyl group is required for mutation induction (9, 24, 25) and suggests that cytotoxicity is dissociable from mutagenicity. Replacement of the amine linkage with an ether linkage (ICR 283) also yields a mutagenically inactive compound.

Four compounds found to be highly mutagenic (ICR 170, 340, 292, and 342), are carcinogenic, and the other two (ICR 191 and 191-OH) are reported to be noncarcinogenic (30, 31). ICR 191 is a potent mutagen not only in our system but also in several others (8, 20). Apparently, ICR 191 is a mutagenic non-carcinogen. Perhaps its lack of carcinogenicity can be explained by its being inactivated in the animal since the addition of S_9 greatly reduces the mutagenicity and cytotoxicity of this compound (J. C. Fuscoe and A. W. Hsie, unpublished observations).

Metallic compounds (total of 19): As mentioned earlier, cis-$Pt(NH_3)_2Cl_2$ is mutagenic whereas trans-$Pt(NH_3)_2Cl_2$, K_2PtCl_4 and $[Pt(NH_3)_4]Cl_2$ are not mutagenic (19). The mutagenicities of the other 15 metallic compounds were determined, and the preliminary result shows that the carcinogenic metallic compounds $NiCl_2$, $CoCl_2$, $BeSO_4$, $CdCl_2$, $FeSO_4$, $CaSO_4$, $AgNO_3$, $Pb(CH_3COO)_2$, $ZnSO_4$, $K_2Cr_2O_7$ and $MnCl_2$ are mutagenic, whereas the noncarcinogenic metallic compounds RbCl, H_2SeO_3, $TiCl_4$ and $MgCl_2$ are not (5, D. B. Couch, E.-L. Tan, N. L. Forbes, K. R. Tindall, and A. W. Hsie, unpublished).

The results of these studies should be viewed with a caution because metal mutagenesis is sensitively modified by the cellular growth condition. For example, demonstration of the mutagenicity of $MnCl_2$ appeared to depend on the relative concentration of $MnCl_2$ vs $MgCl_2$ in the medium during treatment. With many metals, at high concentrations which caused severe growth inhibition and/or significant cellular lethality, metal mutagenicity was either not demonstrable or much lower than expected from the linear dose-response at low dose-range. Thus, metal mutagenesis appeared to require active DNA synthesis. This is consistent with the notion that metal-mediated enhancement of DNA replicative error may account for one of the mechanism of mutation-induction by metallic compounds (37). Growth inhibition with inactive DNA synthesis during treatment would, therefore, disfavor mutation induced by mutagenic metals.

Miscellaneous compounds (total of 19): Non-mutagenic compounds included: three commonly used solvents (acetone, dimethylsulfoxide, and ethyl alcohol), four metabolic inhibitors (cytosine arabinoside, hydroxyurea, caffeine, and cycloheximide), and N^6, $O^{2'}$-dibutyryl adenosine 3':5'-phosphate. The following 11 compounds are mutagenic: hydrazine, hycanthone, ethylene oxide,

ethylene dibromide, ethylene dichloride, ethylene chlorobromide, mitomycin C, myleran, 5-bromodeoxyuridine, captan, and folpet (E.-L. Tan, P. A. Brimer, J. P. O'Neill and A. W. Hsie, unpublished observations). Some experiments are preliminary in nature and remained to be confirmed.

Structure-activity studies of promutagenic chemicals

The CHO/HGPRT assay can be coupled to the host (mouse)-mediated metabolic activation system (14) or the S_9 microsomal activation system (23). We employed S_9 prepared from Aroclor 1254-induced male Sprague-Dawley rat livers, and B(a)P and DMN to define conditions optimum for S_9 activation. Over an S_9 protein concentration range of 0.1 to 1.5 mg/ml, the mutagenicity of a constant amount of DMN increases with increasing protein, while that of B(a)P increases at low protein concentrations, followed by a decrease. This may reflect a change in the balance between activation and inactivation with different compounds. In addition, DMN requires high concentrations of $CaCl_2$ for maximal mutagenic activity, whereas B(a)P does not. These results complicate the development of a single S_9 protein mix which would be useful in routine mutagen screening and in the quantification of the mutagenic potential of compounds for comparative studies (28).

With this limitation in mind, we have determined the mutagenicity of various classes of promutagens under conditions which are near-optimal for quantifying the mutagenicity of both DMN and B(a)P in which the S_9-mix contains $MgCl_2$ and $CaCl_2$ at 10 mM each (28).

Polycyclic hydrocarbons (total of 27): We have studied the mutagenicity of B(a)P and its 19 metabolites, benzo(e)pyrene [B(e)P], pyrene, benz(a)anthracene,

(BA), and 4 related compounds. The carcinogenic polycyclic hydrocarbons B(a)P, BA, and 7,12-dimethyl-BA require metabolic activation to be mutagenic. The weak carcinogen B(e)P is less mutagenic than B(a)P. The non-carcinogenic polycyclic hydrocarbons pyrene and anthracene are non-mutagenic even with metabolic activation. B(a)P-4,5-epoxide and B(a)P-7,8-diol,9-10-epoxide are mutagenic (A. W. Hsie and P. A. Brimer, unpublished).

Nitrosamines and related compounds (total of 16): Nitrosamines generally require metabolic activation to be cytotoxic and/or mutagenic. All nine carcinogenic nitrosamines (dimethylnitrosamine, diethylnitrosamine, 2-methyl-1-nitrosopiperidine, 3,4-dichloro-1-nitrosopiperidine, nitrosopyrrolidine, 3,4-dichloronitrosopyrrolidine, 1,4-dinitrosopiperazine,1,5-dinitrosohomopiperazine, nitrosomorpholine) are mutagenic, and all four non-carcinogenic nitrosamines (2,5-dimethylnitrosopiperidine, 2,5-dimethylnitrosophrrolidine, 1-nitrosopiperazine, nitrosophenmetrazine) are non-mutagenic. Formaldehyde and sodium nitrite are non-mutagenic, and dimethylamine is mutagenic at high concentrations (J. R. San Sebastian and A. W. Hsie, unpublished). Variable carcinogenicity data on the latter three chemicals exist in the literature.

Quinoline compounds (total of 5): Quinoline, a known carcinogen, is mutagenic when metabolically activated by S_9. Another carcinogen, 4-nitroquinoline-1-oxide, is highly mutagenic; its mutagenicity decreases in the presence of S_9. The carcinogenicity of 8-dihydroxy-, 8-amino-, and 8-nitroquinoline is not known, but these compounds exhibit variably weak mutagenicity in preliminary experiments (J. R. San Sebastian and A. W. Hsie, unpublished).

Aromatic amines (total of 5): The carcinogens 2-acetylaminofluorene and its N-hydroxy and N-acetoxyl derivatives are mutagenic, whereas fluorene, a non-carcinogenic analog, is non-mutagenic. 1-hydroxy-2-acetylaminofluorene

appears to be mutagenic at a very high concentration in preliminary experiments (A. W. Hsie, W. N. C. Sun, and P. A. Brimer, unpublished).

Miscellaneous promutagens (total of 4): Three chemicals which are mutagenic to mice (natulan, cyclophosphamide, and triethylenemelamine) are mutagenic in our system (E. L. Tan and A. W. Hsie, unpublished data). The mutagenicity of the artificial sweetener saccharin appears to be variable; its determination is difficult because high concentrations of the compound are needed to yield any biological effect (J. P. O'Neill, J. R. San Sebastian, and A. W. Hsie, unpublished).

Quantitative Analyses of Radiation-Induced Lethality and Mutagenesis (total of 7 agents

Ultraviolet irradiation does not appear to be detrimental for clonal growth up to ~ 8 J/m^2; it induces mutation effectively with increasing fluence from ~ 2 up to ~ 26 J/m^2 (11, 33). The cells at G_1-S_{boundry} appears to be more sensitive to its mutagenic action (33). Fluorescent white, black and blue lights are weakly lethal and mutagenic. Sunlamp light is highly lethal and mutagenic; these biological effects are demonstrable within 15 sec of exposure under conditions recommended by the manufacturer for human use. Lethal and mutagenic effects were observed in cells after 4 min of sunlight exposure (12). The mutagenicity of high doses of X-irradiation is clearly demonstrable; the precise dose response remains to be established after factors affecting ionizing mutagenesis are clarified (23).

Correlation of Mutagenicity in the CHO/HGPRT Assay with Reported Carcinogenicity in Animal Tests

In a total of 132 chemical and physical agents studied at different stages

of completion, 84 have been reported to be either carcinogenic or non-carcinogenic in animal studies. Mutagenicity in the CHO/HGPRT assay of 77 of these agents correlated with the documented carcinogenicity in animals (18, 40). The existence of a high correlation [77/84(92%)] between mutagenicity and carcinogenicity speaks favorably for the utility of this assay in prescreening the carcinogenicity of chemical and physical agents. Since so far only limited classes of chemicals have been tested and some of the preliminary results remain to be confirmed, this correlation should be viewed as preliminary in nature.

Interactive Effects Between Physical and Chemical Agents

Recently, we have shown that near-ultraviolet light up to 300 J/m^2 and 8-methoxypsoralen up to 20 µg/ml are neither cytotoxic nor mutagenic to the cells. However, the "dose"-dependent cytotoxicity and mutagenicity of each agent when the other is kept at a constant level has been shown recently. Similarly, the mutagenicity of B(a)P in the presence of near-ultraviolet light can be demonstrated independent of S_9-activation (R. L. Schenley and A. W. Hsie unpublished), as shown earlier by others in experiments similar to ours (1).

Applications to Screening for Organic Mixtures and for Industrial Chemicals

We have found that a crude organic mixture derived from a fractionated liquified coal sample is mutagenic in this system when assayed with S_9 (17). E. I. Du Pont de Nemours and Co. has found it useful to screen for chemicals such as vinyl chloride not only in the liquid form, but also in gaseous state (2). Other institutions, such as Allied Chemical Corp., Dow Chemical Co., Carnegie-Mellon Institute of Research, and Chemical Industry Institute of

Toxicology, have set up CHO/HGPRT as an integral part of their toxicological research, development, and screening programs.

A Multiplex CHO Genetic Toxicology System: Simultaneous Determination of Cytotoxicity, Mutation Induction, Chromosome Aberration, and Sister-Chromatid Exchange (SCE) in Mammalian Cells

We have so far shown that CHO cells are useful for studies of the cytotoxicity and mutagenicity of various physical and chemical agents. CHO cells were also found to be suitable for studies of carcinogen-induced chromosome and chromatid aberrations and SCE's.

We have studied the interrelationships among these four distinct biological effects by use of carcinogenic/non-carcinogenic pairs of chemicals. These include the direct-acting carcinogen MNNG and its non-carcinogenic analogue MNG, as well as the procarcinogens B(a)P and DMN and their analogues, pyrene and dimethylamine (DMA), respectively. The carcinogens showed induction of mutation, chromosome aberrations, and SCE's, while the non-carcinogenic agents showed little or no such activities. Cytotoxicity does not appear to correlate with mutagenicity for any of the pairs. On an equimolar basis, pyrene has a toxicity similar to that of B(a)P, DMA is less toxic than DMN, and MNG has minimum toxicity. The frequency of SCE's can be quantified over a lower concentration range than is necessary for the determination of chromosome aberrations and mutation induction (34).

The successful development and validation of the multiplex CHO Cell Genetic Toxicity System will be valuable, because this system will allow the simultaneous determination of four distinct biological effects. Recently, we have found that CHO cells is also useful for determination of mutagen-

induced micronuclei formation, the so-called micronucleus test (35); furthermore, endoduplication of chromosome (J. R. San Sebastian and A. W. Hsie, unpublished) and the HeLa DNA synthesis inhibition test (29) can be adapted to CHO cells in a preliminary study (R. B. Painter, personal communication). Thus, if fully developed and validated, the system can determine these additional end points of genetic toxicity.

SUMMARY AND CONCLUSION

The CHO/HGPRT system has been developed and defined for quantifying mutation induced by various physical and chemical agents at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells. Various genetic, biochemical, and physiological evidence supports the genetic basis of mutation induction in this system.

In all direct-acting chemical mutagens studied, mutation induction increases linearly as a function of the concentration, with no apparent threshold. Some chemicals induce mutation at non-cytotoxic concentrations; others induce mutation only with a concomitant loss of cell survival. In one dosimetry study, the mutagenicity of ethyl methanesulfonate has been quantified as a function of exposure concentration × treatment time. The sensitive and quantitative nature of the system enables studies of the structure-activity (mutagenicity) relationships of various classes of chemicals, including alkylating agents, heterocyclic nitrogen mustards, and platinum compounds. When rat liver S_9-mediated metabolic activation is present, procarcinogens such as benzo(a)pyrene, 2-acetylaminofluorene, and dimethylnitrosamine are mutagenic, whereas their noncarcinogenic structural analogues pyrene, fluorene, and dimethylamine are not. Mutagenicity as determined in the assay appears

to correlate well (77/84=92%) with the reported carcinogenicity in animals of 132 chemicals being examined. Quantification of mutagenicity of pro-carcinogens is complicated by the different optimum activation conditions required for different compounds such as benzo(a)pyrene and dimethylnitrosamine.

The system has been shown to be useful in determining the interactive effects between physical and chemical agents, and in screening for mutagenicity of a fractionated organic mixture derived from a liquified coal sample, and of industrial chemicals such as vinyl chloride in both liquid and gaseous state. For the system to be used successfully in routine screening, further studies should be directed toward the development of a metabolic activation system suitable for a broad spectrum of industrial and environmental chemicals, a sensitive and reliable statistical method well-defined in consideration of specific intrinsic characteristics of the CHO/HGPRT protocol to clearly differentiate mutagenicity from non-mutagenicity, and an experimental design to determine compounds with low, yet detectable, mutagenicity.

The system has been expanded for determination of mutagen-induced chromosome aberration, sister-chromatid exchange, and micronucleus formation in addition to gene mutation and cytotoxicity; it can also be used to study inhibition of DNA synthesis. Development of this Multiplex CHO Genetic Toxicology System should allow simultaneous determination of multiple, distinct biological end points and studies of interrelationships among these effects.

ACKNOWLEDGMENTS

We thank D. B. Couch, N. L. Forbes, J. C. Fuscoe, M. H. Hsie, N. P. Johnson, A. P. Lie, R. Machanoff, J. C. Riddle, and E.-L. Tan for contributing various aspects of the study described in this article.

REFERENCES

1. Barnhart, B. J., Cox, S. H., and Okinaka, R. T. (1978): Mutagenicity and cytotoxicity of light-activated procarcinogens. J. Cell Biol. 79: 388a.

2. Barskey, F. C., Irr, J. D. and Krahn, D. F. (1979): Mutagenicity of gases in Chinese hamster ovary cell assay. In Proc. Annu. Meet. Environ. Mutagen Soc., New Orleans, Mar. 8-12, 1979, p. 89.

3. Chu, E. H. Y. and Powell, S. S. (1976): Selective systems in somatic cell genetics. Adv. Hum. Genet. 7: 189-258.

4. Couch, D. B. and Hsie, A. W. (1976): Dose-response relationships of cytotoxicity and mutagenicity of monofunctional alkylating agents in Chinese hamster ovary cells. Mutat. Res. 38: 399.

5. Couch, D. B. and Hsie, A. W. (1978): Metal mutagenesis: Studies of the mutagenicity of manganous chloride and 14 other metallic compounds in the CHO/HGPRT assay. Proc. Annu. Meet. Environ. Mutagen Soc., San Francisco, Mar. 9-13, 1978, p. 74.

6. Couch, D. B. and Hsie, A. W. (1978): Mutagenicity and cytotoxicity of congeners of two classes of nitroso compounds in Chinese hamster ovary cells. Mutat. Res. 57: 209-216.

7. Couch, D. B., Forbes, N. L. and Hsie, A. W. (1978): Comparative mutagenicity of alkylsulfate and alkanesulfonate derivatives in Chinese hamster ovary cells. Mutat. Res. 57: 217-224.

8. Deluca, J. G., Kaden, D. A., Krolewski, J., Shopek, T. R. and Thilly, W.S. (1977): Comparative mutagenicity of ICR-191 to S.typhimurium and diploid human lymphoblasts. Mutat. Res. 46: 11-18.

9. Fuscoe, J. C., O'Neill, J. P., and Hsie, A. W. (1979): Structure-activity relationship of 19 antitumor ICR compounds in CHO/HGPRT system. In Proc. Annu. Meet. Environ. Mutagen Soc., New Orleans, Marc. 8-12, 1979, p. 112.

10. Hsie, A. W., Brimer, P. A., Mitchell, T. J., and Gosslee, D. G. (1975): The dose-response relationship for ethyl methanesulfonate-induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells. Somat. Cell Genet. 1: 247-261.

11. Hsie, A. W., Brimer, P. A., Mitchell, T. J., and Gosslee, D. G. (1975): The dose-response relationship for ultraviolet light-induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells. Somat. Cell Genet. 1: 383-389.

12. Hsie, A. W., Li, A. P., and Machanoff, R. (1977): A fluence-response study of lethality and mutagenicity of white, black, and blue fluorescent light, sunlamp, and sunlight irradiation in Chinese hamster ovary cells. Mutat. Res. 45: 333-342.

13. Hsie, A. W., Brimer, P. A., Machanoff, R. and Hsie, M. H. (1977): Further evidence for the genetic origin of mutations in mammalian somatic cells: The effects of ploidy level and selection stringency on dose-dependent chemical mutagenesis to purine analogue resistance in Chinese hamster ovary cells. Mutat. Res. 45: 271-282.

14. Hsie, A. W., Machanoff, R., Couch, D. B., and Holland, J. M. (1978): Mutagenicity of dimethyl nitrosamine and ethyl methanesulfonate as determined by a quantitative host-mediated CHO/HGPRT assay. Mutat. Res. 51: 77-84.

15. Hsie, A. W., Couch, D. B., O'Neill, J. P., San Sebastian, J. R., Brimer, P. A., Machanoff, R., Riddle, J. C., Li, A. P., Fuscoe, J. C., Forbes, N. L. and Hsie, M. H. (1978): Utilization of a quantitative mammalian cell mutation system, CHO/HGPRT, in experimental mutagenesis and genetic toxicology. In Strategies for short-term testing for mutagens/carcinogens (ed. B. E. Butterworth), CRC Press, Inc., p. 39-54; also in Chemical Industry Institute of Toxicology (CIIT) Workshop on "Strategies for Short-Term Testing for Mutagens/Carcinogens," Research Triangle Park, N.C., Aug. 11-12, 1977.

16. Hsie, A. W., O'Neill, J. P., Couch, D. B., San Sebastian, J. R., Brimer, P. A., Machanoff, R., Fuscoe, J. C., Riddle, J. C., Li, A. P., Forbes, N. L., and Hsie, M. H. (1978): Quantitative analyses of radiation- and chemical-induced lethality and mutagenesis in Chinese hamster ovary cells, Radiat. Res. 76: 471-492.

17. Hsie, A. W., O'Neill, J. P., San Sebastian, J. R., Couch, D. B., Brimer, P. A., Sun, W. N. C., Fuscoe, J. C., Forbes, N. L., Machanoff, R., Riddle, J. C. and Hsie, M. H. (1979): Quantitative mammalian cell genetic toxicology: Study of the cytotoxicity and mutagenicity of 70 individual environmental agents related to energy technologies and 3 subfractions of a crude synthetic oil in the CHO/HGPRT system. In Symp. Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, Williamsburg, Va., Feb. 21-23, 1978 (to be published by the U. S. Environmental Protection Agency).

18. International Agency for Research on Cancer (1972-1976): Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 1-10, International Agency for Research on Cancer, Lyon, France.

19. Johnson, N. P., Hoeschele, J. D., Rahn, R. O. and Hsie, A. W. (1979): Mutagenicity, cytotoxicity and DNA-binding of Pt(II)chloroammines in CHO cells. In Proc. Annu. Meet. Environ. Mutagen Soc., New Orleans, Mar. 8-12, 1979, p. 85.

20. McCann, J., Choi, E., Yamasaki, E. and Ames, B. N. (1975): Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. U.S. 72: 5135-5139.

21. O'Neill, J. P. and Hsie, A. W. (1977): Chemical mutagenesis of mammalian cells can be quantified. Nature 269: 815-817.

22. O'Neill, J. P., Brimer, P. A., Machanoff, R., Hirsch, G. P. and Hsie, A. W. (1977): A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells: Development and definition of the system. Mutat. Res. 45: 91-101.

23. O'Neill, J. P., Couch, D. B., Machanoff, R., San Sebastian, J. R., Brimer, P. A., and Hsie, A. W. (1977): A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Utilization with a variety of mutagenic agents. Mutat. Res. 45: 103-109.

24. O'Neill, J. P., Fuscoe, J. C. and Hsie, A. W. (1978): Mutagenicity of heterocyclic nitrogen mustards (ICR compounds) in cultured mammalian cells. Cancer Res. 38: 506-509.

25. O'Neill, J. P., Fuscoe, J. C. and Hsie, A. W. (1978): Structure-activity relationship of antitumor agents in the CHO/HGPRT system: Cytotoxicity and mutagenicity of 8 ICR compounds. Proc. Am. Assoc. Cancer Res. 19: 61.

26. O'Neill, J. P., and Hsie, A. W. (1979): The CHO/HGPRT mutagenicity assay. II. Genetic basis of 6-thioguanine resistance. In Banbury Conference. II. Quantitative Mammalian Cell Mutagenesis and Mutagen Screening (ed.) Hsie, A. W., O'Neill, J. P. and McElheny, V. K.) in press.

27. O'Neill, J. P. and Hsie, A. W. (1979): Phenotypic expression time of mutagen-induced 6-thioguanine resistance in Chinese hamster ovary cells (CHO/HGPRT system). Mutat. Res. 59: 109-118.

28. O'Neill, J. P., Machanoff, R., and Hsie, A. W. (1979): The use of a rat liver S_9 activation system in the CHO/HGPRT mutation induction assay. In Proc. Annu. Meet. Environ. Mutagen Soc., New Orleans, Mar. 8-12, 1979, p. 67.

29. Painter, R. B. (1977): Rapid test to detect agents that damage human DNA. Nature 265: 650-651.

30. Peck, P. M., Tan, T. K., and Peck, E. B. (1976): Pulmonary carcinogenesis by derivatives of polynuclear aromatic alkylating agents. Cancer Res. 36: 2423-2427.

31. Peck, P. M., Tan, T. K., and Peck, E. B. (1976): Carcinogenicity of derivatives of polynuclear compounds. J. Med. Chem. 19: 1422-1423.

32. Puck, T. T. (1972): The Mammalian Cell as a Microorganism, 219 pp. Holden-Day, Inc., San Francisco.

33. Riddle, J. C. and Hsie, A. W. (1978): An effect of cell cycle position on ultraviolet-light-induced mutagenesis in Chinese hamster ovary cells. Mutat. Res. 52: 409-420.

34. San Sebastian, J. R., O'Neill, J. P., and Hsie, A. W. (1979): A multiplex CHO genetic toxicology system: Simultaneous determination of cytotoxicity, mutation induction, chromosome aberration and sister chromatid exchange in mammalian cells. In Proc. Annu. Meet. Environ. Mutagen Soc., New Orleans, Mar. 8-12, 1979, p. 111.

35. Schmid, W. (1975): The micronucleus test. Mutat. Res. 31: 9-15.

36. Siminovitch, L. (1976): On the nature of heritable variation in cultured somatic cells. Cell 7: 1-11.

37. Sirover, M. A. and Loeb, L. A. (1976): Metal-induced infidelity during DNA synthesis. Proc. Natl. Acad. Sci. U.S. 73: 2331-2335.

38. Thielmann, H.-W., Schroder, C. H., O'Neill, J. P., Brimer, P. A. and Hsie, A. W. (1979): Relationship between DNA alkylation and specific-locus mutation induction by N-methyl- and N-ethyl-N-nitrosourea in cultured Chinese hamster ovary cells (CHO/HGPRT system). Chem.-Biol. Interact. (in press).

39. Thompson, L. H. and Baker, R. M. (1973): Isolation of mutants of cultured mammalian cells. Methods Cell Biol. 6: 209-281.

40. United States Public Health Service. 1967-1973. Survey of Compounds which have been tested for Carcinogenic Activity, USPHS Publication No. 149.