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TITLE: COMPARISON OF BENZO(A)PYRENE METABOLISM AND MUTATION INDUCTION IN CHO CELLS USING RAT LIVER HOMOGENATE (S9) OR SYRIAN HAMSTER EMBRYONIC CELL-MEDIATED ACTIVATION SYSTEMS

AUTHOR(S): David J. Chen, LS-3
Richard T. Okinaka, LS-3
Gary F. Strniste, LS-5

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LOS ALAMOS SCIENTIFIC LABORATORY
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Comparison of Benzo(a)pyrene Metabolism and Mutation Induction in CHO Cells Using Rat Liver Homogenate (S9) or Syrian Hamster Embryonic Cell-Mediated Activation Systems

DAVID JEN-CHI CHEN, RICHARD T. OKINAKA and GARY F. STRNISTE, Genetics Group, Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

INTRODUCTION

Many carcinogens/mutagens are chemically non-reactive and have to be metabolically activated by cellular enzymes before they can exert their biological effects (1). Many cell types are not capable of activating such procarcinogens. Among these are Chinese hamster ovary (CHO) cells, which are routinely used for studies on mutagenesis. Mutagenesis in CHO cells has been studied by the addition of an enzymatically active liver homogenate (S9) fraction (2). However, the metabolism of procarcinogens, such as benzo(a)pyrene [B(a)P], by rat liver homogenate differs from that in intact cellular activation systems. Consequently, B(a)P-induced mutation frequencies in mammalian cells may vary when different activation systems are used (3,4).

In this study, we are attempting to compare B(a)P metabolism and conjugation in rat liver homogenate (S9 preparation) and in Syrian hamster embryonic (SHE) cells. Furthermore, a CHO mutation assay incorporating either of the activation systems is being used to measure the mutation induction frequency.

MATERIALS AND METHODS

Cell Culture and Mutagenicity Assay

Chinese hamster ovary cells (CHO-AA8-4) were cultured under conditions described elsewhere (5). The protocols for S9 activation and determining cytotoxicity and mutagenicity at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus has been previously reported (2,6). Primary Syrian hamster embryo (SHE) cells were obtained using the protocol of Pienta *et al.* (7). For activation by SHE cells, CHO cells (3×10^5 per 60 mm dish) were co-incubated with lethal irradiated (x-ray, 4000 r) SHE cells (2×10^6/60 mm dish) and B(a)P in αMEM plus 10% fetal calf serum. After 48 h

incubation, cytotoxicity and mutagenicity were determined. The plating efficiencies for non-treated CHO cells were regularly between 90 and 100%. The observed mutant frequency is the ratio of mutant colonies per dish to the number of viable cells plated per dish.

Assay of B(a)P Metabolites

For metabolic activation, S9 protein (0.5 mg/ml), cofactors (2) and 1 µg/ml [^{3}H]-B(a)P were incubated at 37°C. At noted times, one ml samples were removed and twice extracted with 2.5 volumes of ethyl acetate. The samples were eluted with a water:acetonitrile gradient through an Altex reverse phase ultrasphere-ODS column (4.5 x 150 mm) using a Beckman model 334 high performance liquid chromatography (HPLC) system. The flow rate was 1 ml per min; fractions were collected every 15 sec. For the analysis of SHE cell metabolism, cells at 2 x 10^6 per 60 mm dish were incubated with 1 µg/ml [^{3}H]-B(a)P. The resulting metabolites were extracted into ethyl acetate after 24 and 48 h of incubation and were then analyzed by HPLC.

Separation of Conjugated B(a)P Metabolites on Alumina Columns

B(a)P metabolites remaining in the aqueous phase after ethyl acetate extraction were separated by a modification of techniques developed by Autrup (8). The aqueous phases were dried, resuspended in 70% ethanol, and applied to alumina columns. The columns were eluted first with 25 ml of ethanol followed by elutions with 25 ml of water, 50 ml of $(NH_4)_2HPO_4$ (pH 3) and finally with 25 ml of 25% formic acid. Five ml fractions were collected and aliquots were assayed for radioactivity.

RESULTS AND DISCUSSION

Using ethyl acetate extraction methods, the S9 fraction shows rapid water solubilization of B(a)P (> 80% after 2 h incubation) whereas the SHE cells show less than 50% solubilization even after prolonged incubation (24 h) (data not shown). Elution profiles of B(a)P metabolites from HPLC are shown in Figure 1. The results indicate that B(a)P metabolites formed in the S9 preparation differ quantitatively and qualitatively from those formed in SHE cells. For SHE cells (Fig. 1, bottom panel), the major B(a)P metabolite is the 9, 10-diol. The 4,5- (or 7,8-) diols and the 7,8,9,10-tetrols (hydrolysis products of the

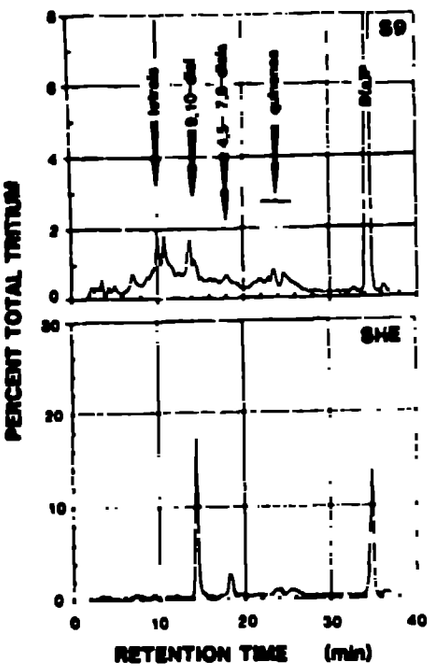

Figure 1. HPLC elution patterns of B(a)P metabolites formed by rat hepatic S9 fraction (top) and SHE cells (bottom). The S9 reactions containing [^{3}H]-B(a)P were extracted with ethyl acetate at various times up to 2 h after the reaction was started. Dishes containing SHE cells were incubated with [3H]-B(a)P for 48 h. The resulting metabolites were extracted into ethyl acetate at various times. Samples of standards of B(a)P metabolites were gifts from the NCI chemical repository.

7,8-diol-9,10-epoxide) are also present in lesser amounts in the medium. In addition to the variety of metabolites mentioned above quinones (primarily the 1,6- and 3,6-) and very hydrophilic derivatives of B(a)P are produced in the S9 preparation (Fig. 1, top panel).

The pattern of B(a)P metabolites produced by x-ray treated (4000 r) SHE cells does not change qualitatively as a function of passage number (up to passage 10, data not shown). This pattern in nonirradiated cells, however, does change with the disappearance of the 9,10-diol and the appearance of more hydrophilic species including tetrols (data not shown).

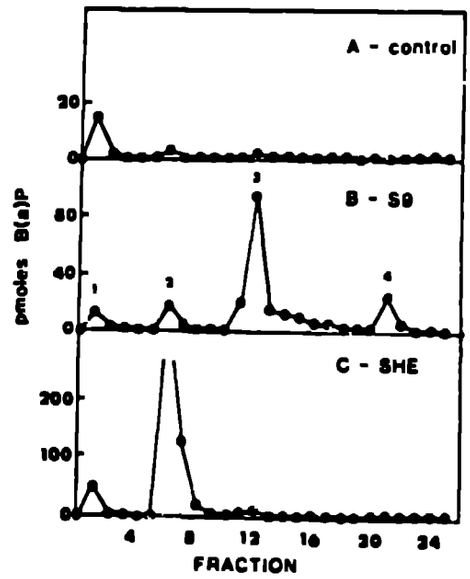

Figure 2. Separation of conjugated B(a)P metabolites on alumina columns (8). The first peak contains primarily non-conjugated B(a)P and some sulfate conjugates. The metabolites in the 2nd, 3rd, and 4th peaks have been presumptively identified as conjugated sulfates-, glucuronides, and glutathiones, respectively. Panel A: B(a)P with no activation; panel B: S9 activation (2 h); and panel C: SHE cell activation (48 h).

Chromatographic separations of conjugated B(a)P species using alumina columns and a 4-step gradient (8) indicate that the two metabolic systems appear to differ significantly in their ability to conjugate B(a)P to sulfates, glucuronic acid, and glutathione. As shown in Fig. 2 (panel C), SHE cells conjugate B(a)P to products which have been presumably identified as being sulfate containing. The S9 reaction appears to contain all three forms of conjugated products (Fig. 2, panel B). These data suggest that the S9 preparations possess a greater potential in removing cytotoxic and mutagenic species by conjugation mechanisms. This notion is consistent with our previous results which showed that at a fixed B(a)P concentration increasing S9 concentrations are followed by the detoxification of cytotoxic and mutagenic events(6).

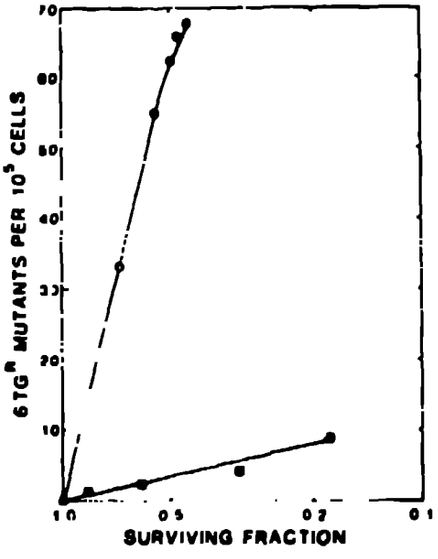

Figure 3. $6TG^R$ mutants induced in CHO cells by B(a)P activated by rat liver S9 (■) or SHE cells (●) as a function of the surviving fraction of cells.

Figure 3 shows the increase in frequency of induced mutations as a function of cell survival after treatment with B(a)P activated by S9 preparation or irradiated SHE cells. At 50% survival SHE cell mediated B(a)P induced mutation frequency is approximately 20-fold higher than that observed with rat liver S9 activation. The lower mutation induction efficiency with S9 activation may be due to its extensive conjugating capacity (Fig. 2). We have also observed that the rate of induction of mutations in CHO is independent of passage number of the x-ray treated feeder layer SHE cells (data not shown). This is consistent with the similar HPLC patterns observed for these x-ray treated cells.

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Key words: HPLC, B(a)P, CHO, Syrian hamster embryonic cell, HGPRT, metabolic activation, cell-mediated activation.