Activation of the Colon Carcinogen 1,2-Dimethylhydrazine in a Rat Colon Cell-mediated Mutagenesis Assay

Carol T. Oravec, Carol A. Jones, and Eliezer Huberman

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

ABSTRACT

Suspensions of rat colon epithelial cells metabolized the potent colon carcinogen, 1,2-[4C]dimethylhydrazine (DMH), into 4C-labeled, alkali-soluble volatile products, presumably CO2. The colon cell suspensions, however, were less effective than hepatocyte suspensions. In addition, we used a cell-mediated mutagenesis assay to test rat colon epithelial cells grown from tissue explants for their ability to metabolize DMH into products mutagenic for human P3 teratoma cells. Mutagenesis in the P3 cells was indicated by an acquired resistance to 6-thioguanine. Co-cultivation of the colon cells with the P3 cells in the cell-mediated assay resulted in mutagenesis, whereas in the absence of the colon cells, no mutagenesis by DMH was observed. Similar results were obtained in a hepatocyte-mediated mutagenesis assay. Colon cells were also able to activate another carcinogen, benzo(a)pyrene, into products mutagenic for the P3 cells. Individual epithelial clonal populations isolated from the colon cultures grown from tissue explants, however, expressed different capacities to activate DMH and benzo(a)pyrene into mutagens, and a high degree of DMH activation by cells from a colon clone was not necessarily associated with a similar degree of benzo(a)pyrene activation. Our results indicate that the colon itself contains epithelial cell types capable of effectively converting DMH into mutagenic (and presumably carcinogenic) products without necessarily involving intermediary metabolism by hepatocytes as previously thought.

INTRODUCTION

Carcinoma of the colon accounts for approximately 10% of cancer deaths each year (1). It is thus imperative to develop and use appropriate experimental systems to investigate mechanisms of colon carcinogenesis. The carcinogen, DMH, which specifically causes colon tumors in experimental animals including the Sprague-Dawley rat (2, 3) is a useful agent for such studies. In the past, it was thought that colon cells possessed insufficient metabolizing enzymes to metabolize DMH to the carcinogenic form. The prerequisite metabolic events were believed to occur in the liver with the active intermediates then transported to the target colon cells via the blood (4).

The present studies were undertaken to re-evaluate this model. In the absence of hepatocytes, we tested the ability of colon cells from the Sprague-Dawley rat to convert DMH into mutagenic metabolites in a cell-mediated assay (5). This type of assay was chosen because it is highly sensitive (6-14) and provides conditions that enable evaluation of the cell/organ specificity of chemical carcinogens (15-21). In the cell-mediated mutagenesis assay, mutable target cells, which cannot themselves metabolically activate many chemical carcinogens including DMH, are cocultured with primary cells derived from a selected organ or tissue. Reactive metabolites generated by the primary cells (in our case, colon cells) are transferred to the target cells where their mutagenic activity can be expressed (13, 21-23). For the present studies we used human teratoma P3 cells as the mutable target cells with resistance to 6-thioguanine used as the genetic marker (24).

Our results indicate that epithelial cells from the colon do have the ability to convert DMH into a mutagen in the cell-mediated mutagenesis assay. Because a reactive mutagenic metabolite is believed to be the ultimate form of a carcinogen, the susceptibility of the colon may be, at least in part, determined by the ability of its cells to activate this organ-specific carcinogen.

MATERIALS AND METHODS

Chemicals. Protease VII, hyaluronidase (type 1), and DMH were obtained from Sigma Chemical Co. (St. Louis, MO), and BP was obtained from Radian Corp. (Austin, TX). The purity of DMH and BP was 99% as determined by using high-pressure liquid chromatography (4). All tissue culture medium supplies were from Grand Island Biological Co. (Grand Island, NY). [14C]DMH (specific activity, 20.5 mCi/ mmol) was obtained from New England Nuclear Research Products (Boston, MA).

Colon Cell Cultures. Colon cells were grown from explant cultures by a modification of a technique developed by Quaroni and May (25). Briefly, colons from germ-free 28-day-old male Sprague-Dawley rats (Charles River Breeding Lab., Wilmington, MA) were resected and rinsed with PBSA containing 1 mm dithiothreitol and then with a cold growth medium that includes Dulbecco's minimal essential medium, fetal calf serum (5%), insulin (10 µg/ml; Collaborative Research, Inc., Waltham, MA), epidermal growth factor (10 ng/ml; Collaborative Research, Inc.), penicillin (100 units/ml), and streptomycin (100 µg/ml). Each colon was then cut into pieces of approximately 5 mm in diameter, and 6 to 8 pieces were placed in each of eight 60-mm tissue culture Petri dishes containing growth medium and incubated at 37°C in a humidified incubator with 5% CO2/95% air. The next day, explants were transferred to new dishes with fresh growth medium containing, in addition, collagenase (200 µg/ml). The following day, the medium was replaced with fresh growth medium containing collagenase (20 µg/ml). The dishes with the explants were then cultured for a period of 6 to 8 wk and refed once per wk with fresh growth medium containing collagenase (20 µg/ml) to restrict fibroblast outgrowth. During this period, epithelial cells grew out from the explants. The primary cultures were obtained after removal of the explants by allowing the cells to reach confluence. Colonies of colon cells exhibiting distinct epithelial morphology were evident within these monolayers. These cells, when analyzed by means of immunofluorescence (26), exhibited a reactivity with a rabbit anti-human keratin antisera (Accurate Chemicals, Westbury, NY) that was similar to the reactivity of cells from a human colon mucosa cell line (DCT Tumor Bank, Worcester, MA). No such reactivity was detected with a rabbit anti-factor VIII antisera that detects an antigen common to endothelial cells (Pel Freez Biologicals, Rogers, AR). A series of these colonies was picked and passaged by scraping the plate after treatment with 0.02% EDTA for 20 min. Some of these isolated colonies would not propagate well upon reestablishment in culture and were not used due to the limited numbers of cells available. Other isolated colonies still contained fibroblast cells and were discarded. Overall, one of about 20 such isolated colonies yielded epithelial cells that could be further subcultured.

Hepatocyte Cell Cultures. Primary hepatocytes were obtained from either conventional healthy bred (4 to 7 wk old) or germ-free male...
Spum-Dawley rats (4 wk old) as described previously (11). The viability of the hepatocytes averaged 90%, as determined by trypan blue exclusion.

Metabolism of \([^{14}C]DMH\). The metabolism of DMH by a suspension of either hepatocytes (prepared as stated above), colon cells [prepared by the method of Shirkey et al. (27)], or P3 cells was determined by measuring the amount of \([^{14}C]\)-labeled, alkali-soluble volatile products, presumably CO\(_2\), generated from \([^{14}C]DMH\) by the cells. In these experiments, \(2 \times 10^6\) cells were incubated for up to 3 h with 60 \(\mu\)M \([^{14}C]DMH\) in 1 ml of growth medium in 20-ml stoppered vials at 37°C in a shaking water bath. Fifteen min prior to the time the reaction was stopped with 7% perchloric acid (0.2 ml), we added 1 ml sodium hydroxide (0.2 ml) to a plastic well suspended from each stopper to absorb the alkali-soluble volatile materials. Following the addition of the perchloric acid, the vials were incubated for another 15 min, after which time the contents of the wells were counted in a liquid scintillation counter (Packard Tri-Carb 300) with ACS cocktail (Amersham Radiochemical).

Colon Cell-mediated Mutagenesis. For the colon cell-mediated assay, we irradiated confluent or near-confluent cultures of primary colon cells or cells from colon clones (1 to \(3 \times 10^5\) cells/60-mm tissue culture Petri dish) with 5000 R of X-rays. After the cultures were irradiated, the growth medium was removed, and the cultures were overlaid with \(3 \times 10^5\) P3 cells in 4 ml of fresh growth medium. Five h later, the cells were treated with a solution of 0.1 ml of PBSA containing DMH or 0.1 ml of growth medium containing BP dissolved in a final concentration of 0.5% dimethyl sulfoxide. The cells were incubated for 48 h with the carcinogen, then dissociated with a solution containing 0.5% trypsin and 0.2% EDTA, and seeded at 200 cells in 5 ml of growth medium in 60-mm tissue culture Petri dishes to determine the cloning efficiencies. The remaining cells were seeded at \(5 \times 10^5\) cells in 10 ml of growth medium in 90-mm tissue culture Petri dishes to allow for optimal phenotypic expression of the mutations. Six to 7 days later, the cells from at least 4 of the dishes were dissociated with trypsin-EDTA, then pooled, and seeded at 200 cells in 5 ml of growth medium in 60-mm dishes to determine cloning efficiencies and at \(2 \times 10^4\) cells in 60-mm dishes in 5 ml of growth medium containing 40 \(\mu\)M TG to select for TG-resistant mutants. The cloning efficiencies were determined 10 days after cell seeding by counting the number of Giemsa-stained colonies in 6 to 8 dishes for each dose of carcinogen. The number of TG-resistant mutants was determined 14 to 16 days after cell seeding by counting Giemsa-stained colonies in 48 to 60 dishes for each dose of carcinogen. Mutation frequencies for TG resistance were calculated per 10\(^6\) colony-forming cells on the basis of the cloning efficiency and the number of cells seeded for mutant selection.

Liver Cell-mediated Mutagenesis. For the liver cell-mediated assay (11), the medium from 25-cm\(^2\) T-flasks containing about \(5 \times 10^5\) P3 cells (seeded 18 h earlier) was removed and replaced with 4 ml of growth medium containing either \(10^5\) or \(10^6\) liver cells. After 4 h, the growth medium was replaced with fresh Leibovitz's L-15 medium containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer; immediately afterwards, 0.1 ml of PBSA containing DMH was added. At this time, the plating efficiency of the liver cells was 65 \(\pm\) 10%. These cells spread over both the P3 cells and occupied spaces in the P3 monolayer to form a contiguous layer. The cultures were incubated for an additional 18 h, after which time the cells were dissociated with trypsin-EDTA to determine the cloning and mutation frequencies as described for the colon cell-mediated assay.

RESULTS

Metabolism of DMH into Volatile Products. In preliminary studies we tested the ability of primary colon cell suspensions to metabolize \([^{14}C]DMH\) into \(^{14}C\)-labeled, alkali-soluble volatile products, presumably CO\(_2\), as a means of determining the overall capacity of the cells to metabolize DMH. For comparison, we included in these studies suspensions of primary hepatocytes and of P3 cells. The P3 cells apparently did not metabolize DMH, because the amount of the alkali-soluble volatile products generated from 60 \(\mu\)M \([^{14}C]DMH\) in the presence of P3 cells was similar to that generated by growth medium in the absence of the cells. The suspensions of both the primary colon and liver cells did, however, metabolize DMH into alkali-soluble volatile products. The amount of \([^{14}C]-labeled\) products generated by colon cells at 3 h after incubation with 60 \(\mu\)M \([^{14}C]DMH\) was one-sixth that generated by hepatocytes (Fig. 1). The increase in metabolite products was both time dependent (Fig. 1) and dose dependent (data not shown).

Colon Cell-mediated Mutagenesis of DMH and BP. Having determined that colon cells were capable of metabolizing DMH, we measured the capacity of the colon cells to generate mutagenic product from this carcinogen. Treatment with 30 to 90 \(\mu\)M DMH of P3 cells cocultivated with colon cells reduced the cloning efficiency of the P3 cells from 78 to 42% in a dose-dependent manner and correspondingly induced TG-resistant P3 cell mutants. The most effective dose of DMH, 90 \(\mu\)M, yielded an 8-fold increase in the mutation frequency. A higher DMH dose, 120 \(\mu\)M, was more cytotoxic but less mutagenic (Table 1).

Since the above experiments were done with primary colon cell cultures, which appeared to be approximately 90% epithelial-like in morphology, we decided to determine if clonal populations derived from these cells would have the same capacity to generate mutagenic metabolites from DMH. DMH treatment of P3 cells cocultivated with cells from four different epithelial clones after their fifth subculture resulted in different degrees of mutant induction in P3 cells (Table 1). Cells from two of these four clones were ineffective in converting DMH at a dose range of 30 to 120 \(\mu\)M into a mutagen for the P3 cells. Cells from one of the other clones activated this carcinogen more effectively than did the primary mixed population of the colon cells, yielding at 30 \(\mu\)M DMH (the lowest DMH dose tested) a mutation frequency at least 10-fold higher than that observed in control cells. The cells from the remaining clone were less effective than the cells from the mixed population in converting DMH into a mutagen. The cells from both responsive clones, as well as those from the mixed population, were most effective...
in activating 90 μM DMH into mutagenic products, whereas the 120 μM dose was less effective. For comparison, we included BP in these experiments. BP, unlike DMH, is a carcinogen with a broader spectrum of activity (Table 1). Colon cells from either the mixed or cloned epithelial cell populations effectively converted BP into a mutagen for the P3 cells, with the different colon cell populations exhibiting different degrees of activity (Table 1). However, no relationship was found between the ability of the different colon cell populations to activate DMH or BP into mutagenic products.

We also examined the ability of hepatocytes to activate DMH in the cell-mediated mutagenesis assay. The results indicate that hepatocytes, like colon cells, can effectively metabolize DMH into mutagenic products for P3 cells and that this activation depends on the number of hepatocytes and dose of DMH (Table 2). Furthermore, the degree of DMH activation by the hepatocytes (Table 2) is similar to that observed for mixed and some cloned colon cells (Table 1).

In short, our results indicate that DMH can be activated into a mutagen by either colon or liver cells.

**DISCUSSION**

Previous investigators have suggested that the colon-specific carcinogen, DMH, is first metabolized to its proximate form, methylazoxymethanol by microsomal mixed-function oxidases in the liver and that this metabolic intermediate is then transported via the blood to the colon where it is further metabolized to the ultimate carcinogenic product (4, 28). Our initial results indicated that colon cells can themselves metabolize DMH into alkali-soluble volatile products, presumably CO₂. Although this metabolism was less effective than that observed in hepatocytes, it nonetheless appears sufficient to produce metabolites that are effective mutagens for the human P3 cells as detected in our colon cell-mediated assay.

Previous studies by Newaz et al. (29), in which human colon microsomes and human colon tumor cells were used, have also shown that colon cells can metabolize DMH. Microsomes from the colons of older persons, who are usually more susceptible to colon carcinogenesis, exhibited a higher degree of DMH metabolism, as detected by DNA alkylation, than those obtained from young individuals (29). Studies by Nyce et al. (30), in which rats were used, have shown a similar pattern of colonic DMH metabolism between young and adult animals. These studies, together with our colon cell-mediated mutagenesis experiments, further substantiate the notion that populations of cells in the colon can metabolize DMH into mutagenic products. Although, clearly, a major portion of DMH metabolism in vivo occurs in the liver, some metabolism can occur in the colon itself. Colon cells possessing this critical metabolic potential may therefore have an increased susceptibility to DMH-induced carcinogenesis because metabolic intermediates generated by the liver are not required.

A number of investigators have suggested that DMH and BP are metabolized by similar enzymatic processes involving the mixed-function oxidases (31). Our cell-mediated mutagenesis experiments with cloned populations of colon cells do not necessarily support this suggestion, because our studies showed no consistent correlation between the ability of a cloned cell population to activate DMH and BP, e.g., effective activation of BP into a mutagen by a cloned population of colon cells was not necessarily associated with a detectable activation of DMH. These latter results indicate that primary colon cultures are composed of a mixed cell population with cells having different functions, including differing capacities to activate certain chemical carcinogens. Other investigators proposed alternative pathways of DMH (and methylazoxymethanol) metabolism involving the following enzymes: alcohol dehydrogenase (32); choline dehydrogenase (33); and prostaglandin synthetase and lipoxygenase (34). It is not yet clear if any or all of these enzymatic systems contribute to colon carcinogenesis. Analysis of these pathways in cloned populations of colon cells with different abilities to generate mutagenic products from DMH in the cell-mediated assay will help to clarify the critical metabolic pathways that result in the conversion of DMH into a mutagen and presumably carcinogen.

In short, our present studies have shown that the colon itself contains epithelial cell types capable of effectively converting DMH into mutagenic (and presumably carcinogenic) products without necessarily involving intermediary metabolism by hepatocytes as previously thought.

**REFERENCES**

7. Hsu, I. C., Stoner, G. D., Autrup, H., Trump, B. F., Selkirk, J. K., and...


Activation of the Colon Carcinogen 1,2-Dimethylhydrazine in a Rat Colon Cell-mediated Mutagenesis Assay

Carol T. Oravec, Carol A. Jones and Eliezer Huberman


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/10/5068

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.