Organ-specific Effects of 1,2-Dimethylhydrazine in Hamster

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Uptake and metabolism of the carcinogen 1,2-dimethylhydrazine (DMH) were compared in isolated epithelial cells from the colon and the small intestine. A new method was developed to separate colonic epithelial cells into surface columnar cells and crypt cells without the use of any proteolytic enzymes. Colonic columnar cell-enriched fraction exhibited DMH metabolism two to three times higher than that of crypt cells. The carcinogen binding was much lower in the small intestine as compared to the colon. In the small intestine, the crypt cell-enriched fraction showed higher carcinogen binding as compared to villus cells. Pyrazole was found to inhibit DMH binding by isolated small intestinal and colonic epithelial cells. The extent of inhibition was maximum in cells showing the greatest ability to incorporate DMH.

Key words: 1,2-Dimethylhydrazine — Colon cancer — Cell culture — Cell isolation

Symmetrical dimethylhydrazine shows a very high degree of organospecificity in hamsters, i.e., nearly all of the tumors after a single injection will be formed in the colon and not the small intestine. The resistance of the small intestine to the carcinogen is quite intriguing considering that the general morphology and physiology of the colonic and intestinal cell populations are quite similar. Transplantation experiments by Gennaro et al.1 and others have shown that the difference in response of these two tissues is due to inherent differences and is not simply a result of exogenous factors such as intestinal flora, bile acid secretion or anatomical location. We are therefore interested in comparing the properties of these two cell populations. Carcinogenesis testing of nitrosamines and nitrosamides have revealed species-specific differences in susceptibility to chemical carcinogenesis.2,3

To date the majority of studies on the carcinogenicity of dimethylhydrazine (DMH) in gut tissue have been conducted in vivo. With the realization that the metabolism of DMH to a reactive species may not require liver participation,4,5 it became clear that much information can be derived by in vitro studies.

Removal of the target tissue (colon) from the influences of the whole animal to a well-regulated culture environment should aid in analysis of the complex factors mediating colon carcinogenesis by DMH. Elucidation of the nature, degree and specificity of chemical carcinogen interaction with different cell types within the gut epithelium may be vital to understanding the extreme organotropism exhibited by colon carcinogens.

Much of the recent knowledge about the mechanism of chemical carcinogenesis derives from the correlation between alkylation of DNA and tumor development or mutation rate.6 Assuming that increased metabolism by an organ can lead to increased alkylation, this investigation was focused on early steps during the interaction of carcinogen with gut epithelial cells — all of which are prerequisites for ultimate ‘initiation’ to occur. Moreover, this represents one of the first attempts to study dimethylhydrazine interactions in vitro with hamster colonic epithelium, and to compare the effects of the carcinogen on susceptible and resistant tissues. Little use has been made of Syrian golden hamsters in colon carcinogenesis studies despite their susceptibility to colonic tumors and the ease of tumor induction by chemical carcinogens.7 A contributing factor may have been the lack of an adequate technique for the isolation of viable epithelial cells from the hamster colon. It has been our

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observation that hamster colonic epithelium does not respond well to cell dissociation techniques used on rat and mouse tissue. Also, to the best of our knowledge, no study of carcinogen uptake and binding by specific enterocyte subpopulations has been performed on any rodent species. Comparisons between proximal and distal colon have not as yet been made because of the small number of cells available in this hamster tissue. Methodology for the dissociation of hamster distal colonic epithelium was developed during the course of this investigation.

**Materials and Methods**

**Cell Isolation and Culture Media** The hypotonic cell isolation medium was composed of 263 ml of Krebs-Ringer phosphate buffer without Mg²⁺ or Ca²⁺, 137 ml of distilled water, 5mM glucose, 300 μg of gentamicin and 0.94% polyvinylpyrrolidone (PVP-40, Sigma). The final osmolarity of the medium was 260 mOsm/liter and its pH was 7.0. The resuspension medium was a solution of Krebs-Ringer phosphate buffer (without Mg²⁺ or Ca²⁺) and containing 0.94% polyvinylpyrrolidone, 5mM glucose, 25mM mannitol and 300 μg/ml gentamicin. This medium was isosmotic (300 mOsm/liter) and had a pH of 7.0. Culture medium was prepared with Leibovitz’s L-15 medium (Gibco, Grand Island, N.Y.) and fetal calf serum (2%). The medium was supplemented with 30 μg/ml of porcine insulin (Sigma), 0.2 μg/ml of hydrocortisone (Sigma), 1.5 μg/ml of pentagastrin (Ayerst Labs), 50 μg/ml of gentamicin sulfate (Schering), 100 U/ml of penicillin (Gibco) and 3 μg/ml of amphotericin B (Gibco).

**Isolation of Colonic Cells** Male Syrian Golden hamsters (about 100 g) were sacrificed by decapitation. The distal colon was removed and its contents were rinsed with a dithiothreitol-saline solution until the sac was maximally distended, the open end was also tied off with a suture. Colonic sacs were washed at room temperature in DTT-saline. They were immersed in a Betadine solution for a few seconds to reduce bacterial contamination. After washing several times in cold DTT-saline, the colonic sacs were transferred to tissue culture flasks (25 cm) containing 20 ml of hypotonic cell isolation medium with 1.5mM ethylenediaminetetraacetic acid (EDTA) and adjusted to pH 7.2. Flasks containing colonic sacs were incubated at 37° for a few seconds to reduce bacterial contamination. After washing several times in cold DTT-saline, the colonic sacs were transferred to tissue culture flasks (25 cm) containing 20 ml of hypotonic cell isolation medium with 1.5mM ethylenediaminetetraacetic acid (EDTA) and adjusted to pH 7.2. Flasks containing colonic sacs were washed at room temperature. They were immersed in a Betadine solution for a few seconds to reduce bacterial contamination. After washing several times in cold DTT-saline, the colonic sacs were transferred to tissue culture flasks (25 cm) containing 20 ml of hypotonic cell isolation medium with 1.5mM ethylenediaminetetraacetic acid (EDTA) and adjusted to pH 7.2. Flasks containing colonic sacs were incubated at 37° in a shaking water bath. Five fractions were collected by incubating the colonic sacs in an isolation medium (IM) with or without EDTA for various times — fraction I, IM + EDTA (20 min); fraction II, IM (10 min); fraction III, IM (5 min); fraction IV, IM + EDTA (10 min); fraction V, IM (30 min). Cells in each of the fractions were passed a number of times through a pasteur pipette and 22 gauge syringe needle to break up cell clumps. Isolated cells were then filtered through bolting silk (No. 8). The filtrate was centrifuged at 4° in an International Clinical Centrifuge at 1000g for 10 min. Cell pellets were resuspended in an ice-cold resuspension medium and stored at 4°. For histological demonstration of graded cell release, colonic sacs were removed at various stages during the isolation procedure. Tissue was quick-frozen in liquid nitrogen and segments (5 mm thick) were mounted on metal holders for cryostat sectioning.

**Carcinogen Uptake** Buffer containing 14C-DMH (3 μCi/100 μl) was added to 200 μl aliquots of epithelial cells. Samples were incubated at room temperature for 0–20 min. The reaction was terminated by adding three volumes of ice-cold Krebs-Ringer phosphate buffer (pH 7.0) and pelleting the cells in an Eppendorf centrifuge. Cells were washed twice with ice-cold buffer and the final cell pellet was dissolved directly in Aquasol for liquid scintillation counting. For zero time values, ice-cold buffer was added directly to the cells, followed by radioactivity measurement.

**Carcinogen Binding** 14C-DMH (11 μCi/ml) was added to 35 mm Corning tissue culture dishes containing epithelial cell suspensions and culture media. Dishes were placed in an incubator at 37° for 1–5 hr. For inhibition studies, a parallel series of dishes was inoculated with carcinogen and the inhibitor pyrazole (1500 μg/ml, Sigma). Contents of dishes were harvested by centrifugation at designated times and supernatant containing the radiolabel was aspirated off. Cells were washed and resuspended in fresh buffer. Duplicate samples...
were filtered onto Whatman GFC filters supported on a Millipore multi-well filtration apparatus and washed under suction (three times) with cold buffer. The filters were further washed in cold 10% trichloroacetic acid (TCA), 0.1 N HCl and ethanol. Filters were allowed to air-dry before liquid scintillation counting. Trapping of counts by the filter itself was corrected by subtraction of the zero time counts.

**Thymidine Incorporation** [Methyl-3H]thymidine (51 Ci/mmol) purchased from ICN (Irving, Calif.) was used. Cell cultures were incubated at 37°C with 1.5 µCi/ml radiolabel. Cultures were harvested at various time intervals. Samples were collected on filters and processed for liquid scintillation counting as described above.

**RESULTS**

**Cell Isolation** While a combination of osmotic swelling and mechanical vibration was shown by Carter et al.8) to be highly effective in eliciting single cell release from the hamster small intestinal epithelium, similar conditions did not promote tissue dissociation in the hamster colon. Disruption of colonic epithelial tissue was found to require the presence of a chelating agent such as EDTA (1.5 mM) in the isolation medium. At the same time, keeping the overall exposure time of colonic tissue to EDTA at a minimum was critical for attaining good cell viability. This technique also avoids the use of proteolytic enzymes. Thus, it should maintain the original surface characteristics of the cell surface involved in attachment to the substratum and in intercellular communication.12-14) Unlike in conventional tissue dissociation techniques developed for rat and mouse colon, where cell isolations were carried out in isotonic media, release of cells from hamster colonic epithelium was best achieved under hypotonic conditions. We observed that under isotonic incubation conditions, a total exposure time of tissue to EDTA of three hours or more was necessary to achieve adequate cell release. The adverse effect of such prolonged incubation on cell viability was evidenced by phase microscopy of cell suspensions. Isolated cells exhibited considerable cytoplasmic blebbing and were stained lightly with Trypan blue, both of which are early indications of cell damage. The use of a slightly hypotonic cell isolation medium (260 mOsm/liter) expedited tissue dissociation, shortening the total time necessary for extensive cell release to less than two hours. This significantly improved cell viability, as did the inclusion of 0.94% polyvinylpyrrolidone in the isolation buffer. Polyvinylpyrrolidone is thought to act by binding compounds released by damaged cells, which may be injurious to viable cells in the preparation.

Under the above conditions, approximately 95% of the released epithelial cells excluded trypan blue, indicating preservation of membrane integrity during the isolation procedure. Another parameter used to assess cell viability was the ability to incorporate radioactive precursors. The pooled cell fractions showed a steady increase in incorporation of labeled thymidine into acid-insoluble material up to five hours, after which levels of incorporation started to fall (Fig. 1).

To address the question of cell population-specific differences, it was imperative that we be able to fractionate isolated cells into at least two major categories — the mature differentiated surface enterocyte (columnar cell) and the undifferentiated mitotically active crypt cell. Carter et al.15) using hamster small intestinal tissue, noted that cell release from the epithelium was graded. The ratio of differentiated to undifferentiated epithelial

![Fig. 1](image_url)  
**Fig. 1.** Ability of colonic epithelial cells to incorporate thymidine in vitro. Isolated colonic epithelial cells were incubated for various time intervals with 3H-thymidine. Incorporation into TCA-precipitable material was measured. Cells were maintained in glass Petri dishes in a CO2 atmosphere for the specified time.
cells decreased in sequentially collected cell fractions. It was necessary to establish the nature of cell release from the colon. In Fig. 2 (a–f), the histology of the colonic epithelia at various stages of dissociation is shown. Cell release from this tissue also appears to be gradual, starting with the surface epithelial cells and progressing towards the crypt bases. Phase-contrast micrographs of representative cell types obtained by this isolation procedure are shown in Fig. 3. The initial fractions (I, II) were composed primarily of long, extremely thin columnar cells with distinctive brush borders, derived from the surface epithelium and also the shorter, more triangular cells of the upper crypt region (fraction III), also exhibiting brush borders. Cuboidal or spherically shaped epithelial cells typical of the lower crypt region predominated in the final fractions (IV, V, Fig. 3g–i). This grada-
of cell release was further corroborated by in vitro thymidine incorporation data. Table I summarizes the incorporation of tritiated thymidine by five sequentially isolated fractions of colonic enterocytes. It can be seen that the specific activity of \(^3\)H-thymidine incorporation increased in successive fractions, coinciding with the increased presence of mitotically active cuboidal crypt cells.

**DMH Studies** In all our carcinogen studies, emphasis was placed on comparing and contrasting the uptake and metabolism of dimethylhydrazine by tissue highly sensitive to its effects (e.g. colon) with those of tissue that is not as susceptible (e.g. small intestine). Figure 4 shows the initial uptake of \(^14\)C-labeled DMH into fresh colonic and small intestinal cell isolates. Radiolabeled carcinogen was added to cell suspensions and the accumulation of radioactivity in the cells was measured as a function of time. Colonic epithelial cells (CEC) took up the carcinogen much more rapidly than small intestinal cells. Within five minutes, almost three times as much carcinogen had been taken up by colonic cells, as shown in the insert of Fig. 4.
Differences in DMH uptake were sustained over the twenty-minute interval studied. Figure 5 contrasts the initial uptake of carcinogen into small intestinal and colonic epithelial cell fractions. In both organs, the uptake of dimethylhydrazine was the greatest in cell fractions enriched in cells incorporating thymidine. From Fig. 5 it can be seen that by twenty minutes, CEC fraction V, which was enriched in cuboidal crypt base cells, had taken up more than twice as much carcinogen as fraction I. The insert in Fig. 5B shows that the increase is apparent in early time periods, suggesting that it is due to faster transmembrane transport. This difference was not quite as marked amongst small intestinal cell fractions.

The amount of label incorporated into TCA-insoluble material was significantly larger in the colon than in the small intestine.

Evaluation of the ability of various cell types within the colon and small intestine to take up dimethylhydrazine and metabolize it to a bound form was an important part of this investigation. Differences in the ability of various fractions to bind DMH were observed. In these studies individual cell fractions from both organs were incubated with $^{14}$C-labeled DMH for five hours. The pattern of carcinogen binding seen in small intestinal cell fractions is shown in Fig. 6. In the upper graph, the level of activity of the intestinal disaccharidase (sucrase) in the individual fractions designated I through V is depicted. This enzyme has been demonstrated to be absent from immature crypt cells and appears only as these cells differentiate and mature into small intestinal villus cells.15) Enrichment of crypt cells in fraction V versus I was confirmed by the decreasing values of sucrase activity. In

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**Fig. 6.** Cell population-specific differences in the ability to bind $^{14}$C-DMH. (A) Small intestine and (B) colon. The specific activity of thymidine incorporated into cells was used to assess the degree of enrichment of crypt base versus columnar cells in the colon. Sucrase activity was used as a marker for the villus cells in the small intestine. The labeling of cells with DMH was performed as described in "Materials and Methods."
the lower graph, changes in the levels of bound radioactivity as fractions were progressively depleted of villus cells and enriched in the crypt cell population are shown. Crypt cell-enriched fractions (IV, V) exhibited 2- to 3-fold higher carcinogen binding than villus cell-enriched fractions (I, II).

Table I shows the increase in alkylation by DMH expressed per µg DNA in relation to thymidine incorporation by the same cells. While DMH metabolism appeared to be correlated with thymidine incorporation in small intestinal cells, this was not the case with colonic tissue. Figure 6 also shows the specific activity of 3H-thymidine incorporation by colonic epithelial cell fractions and the corresponding levels of DMH binding. Maximum binding with radioactive DMH was consistently shown by CEC fractions I and II, both of which incorporated the least thymidine. DMH metabolism by these fractions was 2–3 times greater than that of fractions IV and V, and accounted for approximately 61% of the total DMH bound. Overall carcinogen binding was much higher in colonic than in small intestinal tissue. Note that only the crypt cell-enriched fractions from the small intestine had levels of DMH binding approaching that in colonic tissue.

In Fig. 7, DMH binding by isolated small intestinal and colonic epithelial cells treated in vitro with the inhibitor pyrazole is compared with that of untreated controls. Preliminary results indicated a correlation between sensitivity to pyrazole and ability to bind DMH. In both organs, the maximum inhibitory effect of pyrazole was exerted on those cell fractions enriched in cells best able to bind DMH.

DISCUSSION

The basis for the remarkable organo-specificity of DMH has not yet been clearly established. The fact that tumors do not occur at the site of administration suggests that
dimethylhydrazine requires metabolic activation and/or that not all cells are equally susceptible to the compound, or both. Demonstration of in vitro binding of carcinogen by isolated hamster enterocytes from both the small intestine and colon in this investigation supports the contention of Autrup et al.4,5) that DMH reactivity is not solely dependent on preprocessing of carcinogen by the liver. These organs apparently do possess the ability to activate DMH on their own. Whether or not all the activation steps are identical to those shown to occur in vivo.16-18) cannot be stated definitively. Distinct differences were noted between the small intestine and colon in overall ability to take up and metabolize dimethylhydrazine. Colonic epithelium as a whole carried out both processes more efficiently than small intestinal tissue. What is intriguing is the divergence of the ability to take up DMH from the ability to metabolize DMH to a bound form seen with colonic cell populations. This was in contrast with the situation in the small intestine, where cells taking up the most carcinogen also exhibited the highest binding, whereas colonic columnar cell-enriched fractions showed DMH metabolism two to three times higher than that of crypt cells, despite a much greater initial rate of DMH uptake in crypt cell-enriched colonic fractions.

It was difficult to reconcile the greater efficiency of colonic crypt cells at taking up DMH with the greater ability of the more differentiated enterocytes to bind DMH without considering the possibility of cell-specific differences in enzymes necessary for DMH activation. Results from studies by Grab and Zedeck,19) using cytosolic preparations from the liver and colon, have indicated participation of NAD-dependent dehydrogenase(s) in the metabolic processing of MAM to the methyldiazonium ion. Moreover, pyrazole, a potent inhibitor of dehydrogenase activity, effectively blocked the enzymatic dehydrogenation of alcohol.20) In other studies, in vivo administration of pyrazole was shown to alter azoxymethane metabolism21) and alkylation of cellular DNA.22) The possibility of cell type-specific differences in enzymes was tested in vitro by using pyrazole. It was reasoned that if an alcohol dehydrogenase-like enzyme is important in the ultimate activation of DMH, then tissue and cell populations binding the most carcinogen should exhibit the greatest inhibitory response to pyrazole treatment. This was indeed the case. This would imply that the enhanced ability of particular cell types to undergo alkylation by DMH is related to the presence in these cells of enzyme(s) which facilitate DMH metabolism. Feinberg and Zedeck23) have preliminary data indicating that although MAM can spontaneously decompose, the alcohol dehydrogenase-mediated reaction produces the reactive methyldiazonium ions at a faster rate. While alcohol dehydrogenase itself is probably not involved in the metabolism of DMH, as shown by Fiala et al.,24) other similar dehydrogenases probably act on this compound.25) Interestingly enough, investigators were unable to detect NAD-dependent dehydrogenase activity in cytosolic preparations from tissues relatively resistant to tumor formation, such as kidney and small intestine. To date, no such tissue specificity has been discovered for any other step in the DMH activation pathway.

Currently, studies are being conducted in our laboratory to investigate the enzyme distribution of the villus and crypt cell populations in both tissues, the small intestine and the colon. Attempts are also being made to identify the metabolic pathway of DMH in the small intestine and the colon using HPLC techniques. Whether or not its apparently enhanced ability to take up and metabolize the carcinogen imparts a greater sensitivity of colonic tissue to DMH-induced carcinogenesis is difficult to say. More than likely, this property is simply one of the many peculiarities of colonic tissue which, taken together, ultimately render it more susceptible to malignant transformation. One must remember that in whole individuals, and in organs, it is the balance of final activation at a susceptible site, and the repair mechanisms, which determines the final susceptibility, both in the individual and the organ.

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REFERENCES


