Mucosa-preferential DNA Adduct Formation by 2-Amino-3-methylimidazo-[4,5-f]quinoline in the Rat Colonic Wall\textsuperscript{1}

Alichiro Kajikawa, Atsushi Umemoto,\textsuperscript{2} Kunimi Hamada, Masaki Tanaka, Takemi Kinouchi, Yoshinari Ohnishi, and Yasumasa Monden

The Second Department of Surgery [A. K. A. U., K. H., M. T., Y. M.] and Department of Bacteriology [T. K., Y. O.], School of Medicine, The University of Tokushima, Kuramoto-cho, Tokushima 770, Japan

ABSTRACT

The mechanism of mucosa-specific formation of DNA adducts, which was found recently in human intestines, was studied in male F344 rats treated with 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). There are three conceivable pathways for p.o. administered IQ to reach the target colonic mucosal cells: pathway 1, through the digestive canal which exposes from the luminal direction; pathway 2, following enterohepatic circulation re-expose from the luminal direction; and pathway 3, exposure via blood circulation. To investigate these possible pathways, the following surgical procedures were performed: (a) portal catheterization for IQ administration to eliminate pathway 1 and (b) choledochal catheterization for bile drainage to eliminate pathway 2. When both procedures were performed: (a) portal catheterization for IQ administration to eliminate pathway 1 and (b) choledochal catheterization for bile drainage to eliminate pathway 2. When both procedures were combined, only pathway 3 is active. Four types of IQ-DNA adducts were commonly observed in the colons of all experimental groups, with no qualitative difference between the mucosal and muscular layers. When IQ-HCl was administered by p.o. gavage at a dose of 100 \(\mu\)mol/kg body weight, approximately 70\% of the IQ-DNA adducts in the colon mucosa (13.1 \(\pm\) 4.3 adducts/10\(^4\) nucleotides) was induced through pathway 1. Pathway 3 induced the remaining 30\% of mucosal adducts, producing equal adduct levels in both layers. Pathway 2 did not work for adduct formation. The DNA adduct formation was unaffected in the presence of intestinal flora, indicating that detoxification IQ does not reactivate by floral enzymes. In conclusion, mucosa-specific DNA adduct formation in the colon is caused most likely by the absorption of carcinogens through the lumen.

INTRODUCTION

The incidence of colon carcinoma has been increasing during the last few decades in Japan, and several etiological studies support the idea that the westernization of Japanese dietary habits, like high calorie intake or high levels of daily fat and meat consumption and less fiber intake, are linked profoundly to human colon carcinogenesis (1, 2). On the other hand, the existence of a variety of carcinogens in our daily foods, such as mycotoxins, plant alkaloids, food additives, pesticides, nitrosamines, polycyclic aromatic hydrocarbons, and heterocyclic amines, has been established (3–5). These carcinogens are able to induce various DNA modifications and subsequently induce the critical changes of genetic information that generates cancer (6, 7). It is reasonable to assume that the formation of carcinogen-DNA adducts is always occurring in the human body, but the detection of specific carcinogen-DNA adducts in humans is quite difficult because individual levels are extremely low.

In a recent study, we found the existence of several covalent DNA adducts in the human colon and small intestine by highly sensitive \([\gamma-32P]ATP (\times 7000 \text{ Ci/mmol})\) was obtained from ICN Radiochemical (Irvine, CA). Polyethyleneimine-cellulose sheets (POLYGRAM CELL 300 PEI) were purchased from Machery-Nagel (Düren, Germany).

MATERIALS AND METHODS

Chemicals. IQ, streptomyclin sulfate, neomycin sulfate, and bacitracin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RNase A, RNase T1, micrococcal nuclease, and spleen phosphodiesterase were purchased from Worthington Biochemical Co. Ltd. (Freehold, NJ). T4 poly-nucleotide kinase and four kinds of deoxyribonucleoside 3',5'-bisphosphates were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Proteinase K, apyrase, and prostatic acid phosphatase were purchased from Sigma (St. Louis, MO). \([\gamma-32P]ATP (\times 7000 \text{ Ci/mmol})\) was obtained from ICN Radiochemical (Irvine, CA). Polyethyleneimine-cellulose sheets (POLYGRAM CELL 300 PEI) were purchased from Machery-Nagel (Düren, Germany).

Animals. Male Fischer 344 rats (6–7 weeks old) were purchased from NSLC Co. (Hamamatsu, Japan) and housed in polycarbonate cages, two to three animals per cage, for 2 weeks before use in the pathogen-free room of the animal facilities. They were kept under constant conditions of temperature (22 \(\pm\) 2°C) and humidity (55 \(\pm\) 5\%) with a 13-h light/11-h dark cycle. They were fed a commercial diet MF\textsuperscript{TM} (Oriental Yeast, Co., Ltd., Tokyo, Japan) and provided tap water ad libitum.

Antibiotic Treatment. Rats were treated with antibiotics to eliminate microflora from the GI tract. The mixture of streptomycin sulfate, neomycin sulfate, and bacitracin (200 mg each/kg of body weight) was diluted in 1 ml of sterilized saline and given to the rats every 12 h by gavage for 5 days as reported previously (10). The bacterial counts of the large intestine contents of normal rats and the antibiotic-treated rats were usually more than 10\(^{10}\) colony-forming units and less than 10\(^2\) colony-forming units/g, respectively, in anaerobic cultures.\textsuperscript{4} IQ was administered on the third day of the antibiotic course.

Administration of IQ and Rat Surgery. To convert IQ to its HCl salt, 48 mg IQ were dissolved in 0.5 ml of 1 N HCl, then the solution was evaporated under reduced pressure in a rotary evaporator. Rats were given 100 \(\mu\)mol/kg body weight of IQ-HCl diluted with 0.4 ml of sterilized saline. For a single gavage, the IQ-HCl solution was administered into the stomach by injection. For a single administration into the portal vein, the rats were anesthetized with

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\textsuperscript{2}To whom requests for reprints should be addressed.

\textsuperscript{3}The abbreviations used are: MSA, mucosa-specific DNA adduct; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; GI, gastrointestinal; PEI, polyethyleneimine.

\textsuperscript{4}Unpublished data.
Nembutal (50 mg/kg body weight i.p.), and then an abdominal incision was made and a polyethylene catheter was introduced into the mesenteric vein (Fig. 1). The IQ-HCl solution was injected by a microinjection pump (infusion pump SP-10; Nipro, Japan) at a flow rate of 800 µl/h, then the abdominal wall was closed. After IQ-HCl was administered, food and water were given to rats _ad libitum_. For the drainage of bile, the common bile duct was cannulated (Fig. 1).

Combinations of the above procedures were used to clarify the mechanisms of IQ exposure to the colon (Table 1). Since IQ is a dietary carcinogen, it initially reaches the stomach. There are three conceivable pathways for intragastric IQ to reach the target site of carcinogenesis (i.e., the colonic mucosal cells): pathway 1, IQ passes through the small intestine and reaches the colonic canal, and the colonic mucosal cells are exposed to it from an intraluminal direction; pathway 2, this is through the enterohepatic circulation. In brief, after IQ is absorbed from the GI tract, it is transported via the portal vein to the liver. Then, following several metabolizing processes, IQ and its metabolites are excreted into the duodenum and return to the colonic canal and expose the mucosal cells again from the intraluminal direction; pathway 3, following absorption from the GI tract to the liver, IQ is transported via blood circulation.

In experimental groups 1 and 5, all three pathways were active (Table 1). To eliminate pathway 1, IQ was administered via the portal vein (groups 3 and 6) in which pathways 2 and 3 were active. To eliminate pathway 2, IQ was administered by gavage with external bile drainage (group 2) in which pathways 1 and 3 were active. In group 4, only pathway 3 was active.

### Rat Tissues and DNA Isolation

The rats were killed 48 h after administration of IQ by carbon dioxide asphyxiation, and the large intestines were removed. The intestines were opened longitudinally and immediately rinsed free of their contents using 0.15 M potassium chloride. The mucosal layer was scraped off, and the separated mucosal and muscular layers were stored at −80°C until use. DNA was isolated and purified according to the methods reported previously, and the concentration was determined spectrophotometrically at A260 to adjust finally to 2 mg/ml (8, 11, 12).

#### 32P-postlabeling of DNA

DNA adducts were detected by 32P-postlabeling analysis with the intensification method (13). Briefly, 10 µg DNA were digested to deoxyribonucleoside 3'-monophosphates with micrococcal nuclease (3 units) and spleen phosphodiesterase (0.3 units) in a total volume of 10 µl of 20 mM sodium succinate/10 mM CaCl2 (pH 6.0) at 37°C for 3.5 h (14). The digest was diluted 2-fold with water. Then, 10 µl of the DNA digest were taken and incubated with 5 µl of the labeling cocktail containing 1.5 µl kinase buffer (300 mM Tris-HCl, pH 9.5, 100 mM MgCl2, 100 mM dithiothreitol, and 10 mM spermidine), 1.5 µl [γ-32P]ATP (−150 µCi/µl), 0.5 µl (5 units) T4 polynucleotide kinase, and 1.5 µl water at 37°C for 1 h. The labeled digest was treated further with 2 µl potato apyrase (20 units/ml) and 1 µl carrier deoxyribonucleoside 3',5'-bisphosphates (2 µg/µl of each) for 1 h at 37°C (14). In the standard 32P-postlabeling method, the DNA digest was first diluted 15-fold with water, and 5 µl of the aliquot were reacted with 10 µl of the labeling cocktail containing 7.5 µl of [γ-32P]ATP (−15 µCi/µl), 1 µl (10 units) T4 polynucleotide kinase, and 1.5 µl kinase buffer. For quantitation of the DNA adduct level, the total nucleotide number was estimated as described previously (13–15).

#### TLC of the Labeled IQ-DNA Adducts

To purify the IQ-bound nucleotides from a majority of normal nucleotides, the 32P-labeled nucleoside bisphosphates were spotted onto PEI-cellulose sheets and developed with 1.7 M sodium phosphate (pH 6.0) at room temperature for 15 h. The origin was cut out and attached to a new PEI-cellulose sheet using a magnet (16) and developed with 2.7 M lithium formate/5.1 M urea (pH 3.5) from bottom to top. This was further developed in 0.96 M LiCl/6.4 M urea/0.4 M Tris-HCl (pH 8.0) followed by 1.0 M sodium phosphate (pH 6.0) from left to right, with a 3.5-cm paper wick. The DNA adducts were visualized by autoradiography (8). The adduct spots were cut out, and their radioactivities were quantitated by a liquid scintillation counter. Relative adduct labeling values under intensification and standard conditions, including intensification factors, were calculated as reported previously (13–15).

### RESULTS

All of the rats undergoing surgery survived in an acceptable condition until they were killed. The body weight of the rats was 221 ± 10 g at the start of the experiments. Increases in body weight...
**Table 2 Determination of IQ-DNA adduct level (RAL × 10^3)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Layer</th>
<th>Spot 1</th>
<th>Spot 2</th>
<th>Spot 3</th>
<th>Spot 4</th>
<th>Spot 5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mucosa</td>
<td>4.6 ± 1.1</td>
<td>4.4 ± 1.9</td>
<td>1.6 ± 1.1</td>
<td>1.8 ± 1.4</td>
<td>0.7 ± 0.2</td>
<td>13.1 ± 4.3</td>
</tr>
<tr>
<td>2</td>
<td>Muscle</td>
<td>1.7 ± 0.7</td>
<td>3.2 ± 1.5</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.2</td>
<td>0.4 ± 0.3</td>
<td>3.9 ± 1.7</td>
</tr>
<tr>
<td>3</td>
<td>Mucosa</td>
<td>5.4 ± 1.8</td>
<td>3.9 ± 1.5</td>
<td>1.7 ± 0.8</td>
<td>1.1 ± 0.7</td>
<td>0.7 ± 0.4</td>
<td>12.8 ± 4.7</td>
</tr>
<tr>
<td>4</td>
<td>Muscle</td>
<td>2.0 ± 0.7</td>
<td>1.3 ± 0.6</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>4.1 ± 1.6</td>
</tr>
<tr>
<td>5</td>
<td>Mucosa</td>
<td>2.2 ± 1.0</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>n.d.</td>
<td>3.6 ± 1.4</td>
</tr>
<tr>
<td>6</td>
<td>Muscle</td>
<td>1.5 ± 0.7</td>
<td>0.6 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>n.d.</td>
<td>2.8 ± 1.3</td>
</tr>
<tr>
<td>7</td>
<td>Mucosa</td>
<td>2.6 ± 1.7</td>
<td>0.8 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>0.1 ± 0.1</td>
<td>n.d.</td>
<td>4.4 ± 2.8</td>
</tr>
<tr>
<td>8</td>
<td>Muscle</td>
<td>2.6 ± 2.2</td>
<td>0.8 ± 0.6</td>
<td>0.9 ± 0.7</td>
<td>0.1 ± 0.1</td>
<td>n.d.</td>
<td>4.4 ± 3.6</td>
</tr>
<tr>
<td>9</td>
<td>Mucosa</td>
<td>7.5 ± 1.4</td>
<td>2.7 ± 0.6</td>
<td>1.9 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td>12.5 ± 2.3</td>
</tr>
<tr>
<td>10</td>
<td>Muscle</td>
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<td>1.2 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>n.d.</td>
<td>5.8 ± 0.7</td>
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<tr>
<td>11</td>
<td>Mucosa</td>
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<td>0.8 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>12</td>
<td>Muscle</td>
<td>1.9 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**a** RAL, relative adduct labeling. 
**b** Values represent the mean ± SD. 
**c** n.d., not detected.

were not suppressed by treatment with IQ or antibiotics. Body weight decreased approximately 20 g until killed (48 h) only in rats that had undergone surgery and were given IQ. In rats with bile drainage, 10–15 ml bile were excreted in 48 h. It has been reported that when adult male F344 rats were given 20 mg [2-14C]IQ/kg body weight by p.o. gavage, about 40–50% of the radioactivity was recovered in the urine and about 30–38% was in the feces in the initial 48 h (17). Since the formation of IQ-DNA adducts is supposed to be completed during this period, we selected the 48-h time point after IQ administration to kill the rats.

Four types of IQ-DNA adducts usually were observed in the colons of all of the experimental groups (Fig. 2 and Table 2). There was no qualitative difference in IQ-DNA adducts between the mucosal and muscular layers. Colonic DNA from untreated rats showed only two faint DNA adduct spots in the intensification method. Liver DNA from the group 1 rats was also analyzed using the standard 32P-postlabeling method to estimate the intensification factor of the 32P postlabeling, since the colonic DNA adduct level was too low to analyze using this method (13). Both intensification and standard methods gave the same DNA adduct pattern (Fig. 3). The calculated intensification factor values (n — 4) were 6.1 ± 2.0, 15.1 ± 5.2, 2.4 ± 0.2, 7.5 ± 1.8, and 3.1 ± 1.4 for IQ adducts 1, 2, 3, 4, and 5, respectively.

In group 1, the total level of IQ-DNA adducts was significantly higher in the mucosal cells (13.1 ± 4.3 adducts/10^7 nucleotides) than in the muscular layer cells (3.9 ± 1.7) (P = 0.012 by Student’s t-test; Table 2). In group 2 (bile drainage), total adduct levels were 12.8 ± 4.7 in the mucosal cells and 4.1 ± 1.6 in the muscular layer cells. The former was significantly higher than the latter (P = 0.017), and there was no difference between groups 1 and 2. With portal vein administration, both groups 3 and 4 showed no significant differences in total adduct level between the mucosal and muscular layers. The total adduct levels were the same as those of the muscular layer with gavage administration (groups 1 and 2).

Both the normal intestinal flora groups and the flora-reduced groups showed the same total adduct levels with both gavage and portal administration (Table 2).

**DISCUSSION**

IQ was isolated initially from broiled sardines using the Salmonella mutagenesity assay (18, 19). IQ was chosen for this study because the carcinogenicity of IQ in the colonic mucosa of the F344 rat has been well established (20). IQ is a component of ordinary cooked foods and cigarette smoke, and therefore we are always exposed to IQ (21–24). The metabolism of IQ in rats already has been well established by

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Fig. 2. IQ-DNA adduct patterns in the mucosal (A, C, and E) and muscular layers (B, D, and F) of the rat colon. A and B, group 1; C and D, group 4; and E and F, untreated control. 32P-postlabeling assays were run under intensification conditions. Chromatograms were exposed to X-ray films at —80°C for 12 h (A, B, E, and F) and for 16 h (C and D). Black arrowheads, IQ-DNA adduct spots. Open arrowheads, DNA adduct spots originally present in rat colon.

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**DISCUSSION**

IQ was isolated initially from broiled sardines using the Salmonella mutagenesity assay (18, 19). IQ was chosen for this study because the carcinogenicity of IQ in the colonic mucosa of the F344 rat has been well established (20). IQ is a component of ordinary cooked foods and cigarette smoke, and therefore we are always exposed to IQ (21–24). The metabolism of IQ in rats already has been well established by
previous investigators (17, 25, 26), and IQ-DNA adducts are detectable by sensitive 32P-postlabeling analysis (27, 28).

Recently, the presence of MSAs was shown in the human digestive tract, including the stomach, small intestine, and colon (8, 9), while muscular layer-specific DNA adducts did not exist. However, the mechanisms of mucosa-specific DNA modification are not known. In the case of IQ, p.o. administration of IQ induces adenocarcinoma in the rat colonic mucosa, but not in its muscular layer (20). Since some investigators have reported that the level of carcinogen-DNA adduct is usually higher in the target organs than in non-target organs (29, 30), we hypothesized that the level of IQ-DNA adducts would be higher in the mucosa than in the muscular layer. Accordingly, when IQ was administered by gavage (group 1), the total level of IQ-DNA adducts was significantly higher (about 3-fold) in the mucosa than in the muscular layer (Table 2), although the modification was mucosa preferential rather than completely mucosa specific.

Some portion of IQ administered by p.o. gavage passes through the small intestine and reaches the intracolonic space without any metabolism. The IQ is absorbed into colonic mucosal cells by active and/or passive transportation systems. It was shown that N-hydroxy and N-acetoxy derivatives of IQ most likely play a major role for the formation of the DNA adduct formation in vivo (28). N-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4,5-f]quinoline and 5-(deoxyguanosin-N2-yl)-2-amino-3-methylimidazo[4,5-f]quinoline have been identified from the reaction of N-acetoxy derivatives of IQ with deoxyguanosin (31, 32). The activities of cytochrome P450s or esterifying enzymes responsible for the activation described above were shown to be present in the rat colon (33–35). Probably IQ, that transported via pathway 1, is activated to reactive intermediates by these enzymes of the colonic mucosal cells and bind with DNA, because there was an apparent difference in DNA adduct levels between gavage (groups 1 and 2) and portal administered groups (groups 3 and 4). The majority of IQ may be absorbed in the small intestine and colon and brought to the liver by the portal blood flow. It has been reported that only a very minor portion of p.o. administered IQ passes through the intestinal tract in its original form and gets excreted in feces (17). Therefore, most IQ is absorbed by the GI tract. Furthermore, it has been reported that after 24 h, approximately 9% of the administered IQ is excreted into the bile, mainly as metabolites, but approximately 6% of the bile-excreted IQ appears as an unmetabolized parent amine (17). This indicates that only a very small portion of the potential DNA-reactive IQ returns to the intracolonic space by enterohepatic circulation. In our study, total DNA adduct levels were almost the same when groups 1 and 2 were compared. Therefore, enterohepatic recirculation does not contribute to IQ-DNA adduct formation in the colonic mucosa.

In groups 3 and 4, in which IQ was administered through the portal vein and the colonic mucosa was not exposed directly to intraluminal IQ, the total adduct level of the colonic mucosa of group 3 (3.6 ± 1.4 adducts/107 nucleotide) was similar to that of the muscular layer of group 1. This suggests that IQ or its metabolites can reach to colonic mucosal cells via blood circulation. The adduct level of the muscular and mucosal layers in group 3 were almost the same. This indicates that the mucosal and muscular cells are exposed equally to IQ or its metabolites via blood circulation. This idea was supported further by the results obtained in group 4. In group 4, the route to the colonic mucosa was limited to pathway 3 (via blood circulation). The total adduct levels of both the mucosal and muscular layers of group 4 were the same, i.e., 4.4 ± 2.8 and 4.4 ± 3.6 adducts/107 nucleotide, respectively. This again indicates that the IQ-DNA adduct formation occurs equally in the mucosal and muscular cells via blood circulation, and that IQ-derived compounds transported by blood circulation does not play a predominant role in adduct formation in the mucosal layer. It was shown in the case of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine that the main bioactivation of the parent amine to its N-hydroxy derivative and N-acetoxy derivative probably occurred in the liver, and these reactive metabolites were transported to the target tissues via the bloodstream and formed their DNA adducts (36). In the case of IQ, however, it is not clear which is the major activating site, the liver or colon, when IQ-DNA adducts of the colon are formed through the blood circulation.

To summarize, when IQ was administered by p.o. gavage, pathway 1 (direct exposure from the intraluminal site) led to the majority of mucosal DNA adduct formation by colonic activating enzymes but did not form DNA adducts in the muscular layer. Pathway 3 (supply via blood circulation) equally produced DNA adducts in both the mucosal and muscular layers. Pathway 2 (enterohepatic circulation) did not appear to transport reactive IQ.

Previous investigators have reported that during enterohepatic circulation, carcinogens detoxified in the liver can be deconjugated by intestinal bacteria to potential carcinogens that can be activated by the mucosal cell enzymes. For instance, glutathione-conjugated 1-nitropyrene oxides given to mice were cleaved stepwise by γ-glutamyl transeptidase, aminopeptidase of the host and, finally, by bacterial cysteine conjugate β-lyase, and could form DNA adducts in the colonic mucosa (10, 37). p.o. Administered IQ has been shown to be metabolized in the liver and excreted into bile as its glucuronide, sulfate ester, sulphamate, N-acetylated IQ, and ring-hydroxylated IQ (17, 25, 26). We compared reactivation mechanisms by bacterial enzymes for such nonreactive IQ metabolites by comparing normal intestinal flora groups with flora-reduced groups (group 1 versus
group 5 and group 3 versus group 6). Differences in the total adduct level were not observed. This indicates that the intestinal flora does not reactivate detoxified IQ metabolites.

What is a plausible mechanism for the mucosa-specific formation of covalent DNA adducts in the human intestinal tract, which are completely absent in the muscular layer? Differences in cellular functions, such as enzymatic activity for activation/inactivation, transport activity of the cell membrane, and DNA-repairing activity, may be responsible for the mucosa-specific modification by the carcinogens of dietary origin. In addition to such metabolic differences, it is conceivable that the mucosal layer is influenced more directly from intestinal contents than the muscular layer. It is clarified in this study that direct exposure to IQ in the intestinal contents is responsible for the majority of DNA adduct formation (70%) in colonic mucosal cells. In addition, transportation of IQ via blood circulation was responsible for forming some adducts (30%) in the colonic mucosa and also formed an equal level of adducts in the muscular layer cells. In conclusion, even though this study is on rodents and of course not directly consistent with human cases, our data suggest that MSAs in the human colon are generated by genotoxic substances from the intestinal contents that act directly from the intraluminal site.

ACKNOWLEDGMENTS

We thank Dr. Seiichi Mimura and Mohammed Jaber Seraj for assisting in preparing the manuscript.

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