Modulation of N-methyl-N-nitrosourea-induced crypt restricted metallothionein immunopositivity in mouse colon by a non-genotoxic diet-related chemical

Eilish T. Donnelly1, Helen Bardwell2, Geraldine Anne Thomas5,6, E. Dillwyn Williams3, Margaret Hoper1, Paul Crowe1, Wilson G. McCluggage1, Michael Stevenson4, David H. Phillips2, Alan Hewer5, Martin R. Osborne5 and Frederick C. Campbell1,7

1Department of Surgery, Cancer Centre, Queen’s University Belfast, Belfast BT2 6BJ, UK, 2Strangeways Research Laboratories, Worts Causeway, Cambridge CB1 8RN, UK, 3Department of Pathology and 4Department of Epidemiology, Cancer Centre, Queen’s University Belfast, Belfast BT2 6BJ, UK and 5Section of Molecular Carcinogenesis, Institute of Cancer Research, Brookes Lawley Building, Cotswold Road, Sutton SM2 5NG, UK

6Present address: South West Wales Cancer Institute, Singleton Hospital, Sketty, Swansea SA2 8QA, UK

7To whom correspondence should be addressed.

Tel: +44 028 90322276; Fax: +44 028 90321811; Email: f.c.campbell@qub.ac.uk

Red meat consumption is associated with endogenous metabolic generation of mutagenic N-nitroso compounds (NOC) and may be implicated in causation of colorectal cancer. Assessment of a biologically relevant dose of NOCs is hampered by imperfect understanding of NOC interactions with other dietary components. This study tests the hypothesis that NOC effects upon mutational biomarkers in mouse colon may be modulated by a non-genotoxic diet-related compound. N-methyl-N-nitrosourea (MNU) and undegraded β-carrageenan (λCgN) were selected as test chemicals, representing a NOC and a non-genotoxic agent, respectively. Study end-points included (i) DNA adduct formation and (ii) metallothionein (MT) crypt restricted immunopositivity indices (MTCRII) which are considered representative of crypt stem cell mutations. Frequency and size of MT immunopositive foci as well as total number of MT immunopositive crypts were assessed. Biologically effective doses of MNU and λCgN were determined in model validation studies and the agents were then tested alone and in combination. Continuous λCgN treatment for 10 weeks induced significantly greater colonic mucosal injury than a drinking water control. In combined treatment regimens, λCgN treatment did not significantly affect MNU-induced DNA adduct formation. However, combinations of λCgN with MNU significantly increased MTCRII in excess of those induced by MNU alone. Recurrent or continuous λCgN regimens had greater interactive effects with MNU upon MTCRII than short-term λCgN treatment. This study has shown that exposure to a non-genotoxic diet-related compound (λCgN) modulates the effective NOC dosimetry for induction of MT crypt restricted immunopositivity.

Introduction

Dietary or lifestyle factors may be implicated in the 20-fold international variance of colorectal cancer (CRC) risk (1). Consumption of red meat promotes formation and colonic excretion of genotoxic N-nitroso compounds (NOCs) (2) and is linked to CRC (3). Non-genotoxic substances from foods or beverages can modulate mutagen effects (4) and may influence the promotion phase of tumour formation (5). Ethanol, for example, is non-genotoxic with pro-inflammatory properties (6) and may enhance NOC-induced carcinogenesis (7). Native carrageenans are non-genotoxic (8) and are used as food additives. At high doses, native carrageenans may have pro-inflammatory properties (9-12) and may have a role in neoplastic change (13). Food grade λ carrageenan (λCgN) is used as a thickener, emulsifier or stabilizer (14).

Cancer risk assessment of mixtures of dietary or lifestyle toxicants is hampered by limited comprehension of chemical additive or interactive effects (15). Robust biomarkers of early events of tumourigenesis, including DNA damage, somatic stem cell mutation and mutant clonal expansion, may facilitate mechanistic studies of dietary effects. O6-methyldeoxyguanosine and 7-methylguanine adduction formation has been demonstrated in human colorectal DNA (16) and provides a measure of N-nitroso compound-mediated DNA damage. O6-methyldeoxyguanosine is pro-mutagenic and induces characteristic GC→AT transition mutations (17) involving K-ras (18). Mutation of K-ras is a key event of human colorectal tumourigenesis (19,20) and may be implicated in a proportion of diet-related cancers. Within the colon, crypt stem cells represent a primary target for genotoxic events and neoplastic transformation (21,22). Biomarkers of specific oncogene mutations in the stem cell fraction of morphologically normal colonic mucosa are lacking. However, frequencies and patterns of oncologically neutral mutations in colonic crypt stem cells may be assessed by an endogenous marker gene assay, such as glucose 6-phosphate dehydrogenase (G6PD) (23,24). Expression of the mutant marker in stem cell clonal descendants produces a phenotypic change throughout an entire crypt. Crypt fission represents a primary mechanism of clonal expansion in the colon (22). Mutant crypt fission may generate a ‘patch’ of two or more contiguous mutant crypts.

Persistent metallothionein (MT) overexpression within single crypts has been tested as a biomarker of colonic crypt stem cell mutation. Jasani et al. found that the frequency and time course of crypt conversion to MT immunopositivity in mice treated with dimethylhydrazine (DMH) was similar to that of the G6PD assay (25). Cook et al. demonstrated a strong correlation between the MT and G6PD assays (r > 0.9) in Balb/c mice treated with N-ethyl-N-nitrosourea (ENU). The MT bioassay has the advantage of use in paraffin fixed tissue sections (26). Stable, crypt restricted immunopositivity for MT thus correlates with an established mutation marker in the mouse colon (G6PD) and is considered to result from a...
mutation affecting expression of the MT gene in a colonic stem cell (26).

Preliminary studies of this project sought suitable test doses of both undergraded λCgN and the synthetic N-methyl-N-nitrosoureia (MNU) for the toxic effects of interest. Effects of λCgN in drinking water were assessed on colonic mucosal inflammation or injury, using a semi-quantitative histological scoring system (27). Dose–response assays of MNU were carried out against O6-methyldeoxyguanosine and 7-methylguanine adduct formation and MT crypt restricted immunopositivity in mouse colon. Individual and combined λCgN/MNU effects were then assessed against these mutational events. A single treatment with MNU was combined with different temporal λCgN exposures. MNU (62.5 mg/kg i.p.) was thus given alone or in combination with single or recurrent short-term or long-term continuous λCgN treatment (1 or 4% in drinking water). Indirect growth effects of dietary interventions may affect toxicological outcomes (28,29). To accommodate these considerations, we assessed the weight gain of individual animals and group consumption of food and fluid in different treatment categories.

Materials and methods

Chemicals and reagents

λCgN (C3889) extracted from two species of seaweed Gigartina acciculae and Gigartina pistillata was purchased from Sigma (Poole, UK). This was not food grade but is described by the manufacturer as essentially pure undergraded λCgN. MNU and dimethylsulphoxide (DMSO) were also obtained from Sigma UK. Mouse monoclonal anti-thorax PT and peroxidase-conjugated swine anti-rabbit antibodies were obtained from Dako Ltd (High Wycombe, UK) (25,26). The primary mouse monoclonal antibody to MT was used in the study reacts with a highly conserved immunoreactive site within the last five to seven amino acids of the N-terminus of the B domain of the MT protein. [methyl-3H]MNU was obtained from Amersham Biosciences (Little Chalfont, UK). This had been assessed as 99.4% pure by HPLC on a Hypersil MOS column using a multi-linear gradient of 1% glacial acetic acid in water to acetonitrile.

Animals and treatment regimes

Female adult Balb/c mice, aged 6-8 weeks, were obtained from Harlan UK Ltd (Bicester, UK). Animals were divided into groups of 5 or 10 and ear punched. Animal groups were placed in stainless steel wire cages, coded according to (Bicester, UK). Animals were divided into groups of 5 or 10 and ear punched. Animal groups were placed in stainless steel wire cages, coded according to semi-quantitative histological scoring system (27). Dose–response assays of MNU were carried out against O6-methyldeoxyguanosine and 7-methylguanine adduct formation and MT crypt restricted immunopositivity in mouse colon. Individual and combined λCgN/MNU effects were then assessed against these mutational events. A single treatment with MNU was combined with different temporal λCgN exposures. MNU (62.5 mg/kg i.p.) was thus given alone or in combination with single or recurrent short-term or long-term continuous λCgN treatment (1 or 4% in drinking water). Indirect growth effects of dietary interventions may affect toxicological outcomes (28,29). To accommodate these considerations, we assessed the weight gain of individual animals and group consumption of food and fluid in different treatment categories.

Assessment of pro-inflammatory effects of λCgN in mouse colon

Three groups of 5 mice received drinking water containing 0 (control), 1 or 4% λCgN continuously for 10 weeks. Colons were retrieved for gross and histological examination on completion of the treatment. 

Assessment of mucosal gross morphology and histology

Mouse colons were opened longitudinally along the full length and faecal material removed with forceps. Colons were pinned to a cork mat, examined at 40× magnification under a dissecting microscope and morphological changes including redness or ulceration were recorded. Colons were then ‘Swiss-rolled’ on a cork mat, placed in plastic cassettes, fixed in neutral formal-buffered saline for 48 h and embedded in paraffin blocks. Sections of 4 μm were cut at 10 levels 100 μm apart throughout the block and stained with haematoxylin and eosin for histological assessment of crypt loss, crypt hyperplasia or inflammation. Light microscopy of the entire length of all colons was carried out using a Zeiss Axiom microscope, by two investigators (E.T.D. and W.G.McC.) working independently. Consensus scores were given as results. Histological scoring of crypt loss, hyperplasia/distortion and inflammation was carried out as described by Cooper et al. (27) and was blind to treatment category. The total number of crypts were counted in one section per block and the percentage of crypts affected by injury, inflammation or hyperplasia/distortion were scored as follows: score 1, 1-25%; score 2, 26-50%; score 3, 51-75%; score 4, 76-100% (27).

Assessment of MNU effects on colonic DNA adduct formation

Two groups of 5 female mice received 62.5 or 125 mg/kg unlabelled MNU by i.p. injection. Mice were killed after 24 h, colons retrieved and DNA isolated for analysis. Methylated bases were not detectable in mouse colon after treatment with unlabelled MNU. The minimum level of O6-methyldeoxyguanosine detectable by its UV absorption was 20 ng in 1 mg DNA (20 p.p.m.). Tritium labelled MNU ([methyl-3H]MNU) (16.7 Ci/mmol, 161 Ci/g, 5 Ci/l) was used in subsequent experiments. The minimum detectable level of O6-methyldeoxyguanosine using [methyl-3H]MNU was 3 p.p.m. [Methyl-3H]MNU was added to unlabelled MNU for batch treatment. To produce tritiated MNU at doses of 62.5 or 125 mg/kg, 50 μl [methyl-3H]MNU (250 μCi) were added to 6.25 or 12.5 mg unlabelled MNU for each treatment group and diluted according to individual animal weight.

Isolation of DNA

Each colon was homogenized in 1 ml of water containing 10 mM EDTA, 1% SDS and 0.1% proteasine K and incubated at 37°C for 2 h. The RNA content of the isolated material was estimated from peaks due to guanosine and adenosine in the HPLC analysis and amounted to ~5%. Homogenates were extracted with phenol:chloroform (1:1) three times, the DNA precipitated by addition of ethanol, redissolved, treated with RNase A + T and further extracted twice with chloroform:isoamyl alcohol (24:1) (30). DNA was precipitated with ethanol, washed with ethanol and ether, dried and redissolved in 0.5 ml of water.

Analysis of DNA

DNA was hydrolysed to nucleosides by successive treatment, all at 37°C, with: (i) 3 μl DNase I (Roche, 50 U/μl) + 10 μl buffer (0.5 M Tris, pH 7 + 0.5 M MgCl2) for 17 h; (ii) 3 ml snake venom phosphodiesterase (Sigma Type II, 5 U/ml) + 80 μl buffer (0.5 M Tris, pH 9) for 7 h; (iii) 3 ml alkaline phosphatase (Sigma Type III, 300 U/ml) for 17 h. Hydrolysis appeared complete. Incomplete hydrolysis would lead to tritium activity coming straight through the column (before the deoxycytidine peak). Negligible activity was observed in this column. An aliquot of 5 μg O6-methyldeoxyguanosine (Sigma) was added as a marker and the mixture was analysed by HPLC on a Nucleosil ODS column (5 μm, 250 × 4.6 mm) with a (A = 0.05 M ammonium formate, pH 6) to methanol linear gradient (10 min A, then to 30% methanol in 35 min at 0.7 ml/min). The column separated deoxycytidine (15–18 min), thymidine (33 min), deoxyguanosine (35 min), deoxyadenosine (44 min) and O6-methyldeoxyguanosine (50 min). The retention times of 7-methylguanine and 3-methyladenine were 32 and 43 min, respectively. Fractions were collected and assayed for UV absorbance and tritium activity. No other methylated substances are known to co-elute with 7-methylguanine or O6-methyldeoxyguanosine. Absorbance figures were used in the calculation of amounts of each nucleoside present. Tritium activity was counted in fractions with retention times of 23–62 min, where methylated products might be expected. Counting efficiency was 42%. Background activities in unlabelled and labelled samples were 10 and 13–17 c.p.m., respectively. Samples were analysed individually. Analysis of methylated DNA was against standards obtained by treating calf thymus DNA with [methyl-3H]MNU, as previously described (31). 7-Methylguanine and the promutagenic adduct O6-methyldeoxyguanosine were assayed in total DNA and expressed as adducts/106 m.
(mouse anti-horse monoclonal E9, isotype IgG1, 100 μl/slide) (DAKO Ltd, Ely, UK) at a dilution of 1 in 1000 at 4°C overnight. This antibody recognizes both isoforms of the MT protein and is directed against a highly conserved epitope. Negative control sections were incubated either in the absence of antibody, in normal mouse serum (1:1000) or with an irrelevant antibody of the same subclass (1:100) IgG1. These were consistently negative. Positive control sections were of mouse colon previously treated with 250 mg/kg ENU, which induces MT immunopositive crypts (26). Primary antibody was carefully washed off with PBS. Secondary antibody (rabbit anti-mouse immunoglobulin; Dako catalogue product code P0161) was added (100 μl per slide) and incubated at room temperature for 1 h. Sections were then washed, incubated with the chromogen 3,3-diaminobenzidine tetrahydrochloride, counterstained with Harris haematoxylin and mounted with Distere Plasticiser Xylene as previously described (26). One additional section from levels 1, 5 and 10, respectively, were also stained with haematoxylin and eosin. Sections from levels 1 and 10 were used to confirm comparability of the size of the colon section examined between levels and for assay of total crypt number. The number of crypt profiles in one median section per mouse was counted by microscopy incorporating an eyepiece graticule, along a grid of parallel lines 700 μm apart. The number of crypts per section was multiplied by the number of levels studied (n = 10) to determine the total crypt count per mouse, as previously described (26). Dark brown staining crypts against a haematoxylin background were recorded as positives in transverse or longitudinal sections through the crypt lumen. The numbers of adjacent mutant crypts in immunopositive mutant patches were counted and recorded as doubles, triples or greater. Only wholly involved MT immunopositive crypts or patches were regarded as biomarkers of stem cell mutational events. Each single MT immunopositive crypt or patch was considered to represent a single mutant focus. The frequency of MT immunopositive events. Each single MT immunopositive crypt or patch was regarded as biomarkers of stem cell

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Dose selection for combined treatment studies

Statistical modelling was based on previous work using DMH, with or without 6% underaged carrageenan in the diet (32). That study showed an ~80% increase in experimental colonic tumour formation with the combination treatment compared against DMH alone (32). In the present study, 62.5 mg/kg MNU induced sufficient crypt mutations (see Results) to provide the statistical power for detection of an additive or interactive ACgN effect of this magnitude, in 10 animals per group.

Combined treatment studies

Assessment of effects of ACgN on MNU adduct formation. Three treatment groups of 5 mice each received a single i.p. dose of 50 μCi [methyl-3H]MNU with or without 25 μCi [3H]MNU, representing an exposure dose of 62.5 mg/kg with either distilled drinking water only or 1 or 4% ACgN in the drinking water. MNU was administered in 5% DMSO i.p. after 5 days treatment with ACgN. Four control groups of 2 mice each received drinking water only or 1 or 4% ACgN in the drinking water or 5% DMSO vehicle only i.p. Mice were killed 24 h after MNU treatment and colons retrieved for assay of adduct formation by the methods outlined above.

Assessment of ACgN effects on MNU-induced colonic crypt mutation. Eleven groups of mice received no MNU, MNU alone or in combination with 1 or 4% ACgN, as follows (Figure 1): Group 1, drinking water for 10 weeks; Group 2, a single i.p. injection of DMSO, then drinking water for 10 weeks; Group 3, continuous 1% ACgN for 10 weeks; Group 4, continuous 4% ACgN for 10 weeks; Group 5, 62.5 mg/kg MNU i.p. and drinking water for 10 weeks; Group 6, 62.5 mg/kg MNU i.p. and 1% ACgN for 7 days during week 1, then drinking water until 10 weeks; Group 7, 62.5 mg/kg MNU i.p. and 4% ACgN for 7 days during week 1, then drinking water until 10 weeks; Group 8, 62.5 mg/kg MNU i.p. and 3 day treatments with 1% ACgN during weeks 1, 4 and 7, with drinking water given between and after ACgN treatments until 10 weeks; Group 9, 62.5 mg/kg MNU i.p. and three 7 day treatments with 4% ACgN during weeks 1, 4 and 7, with drinking water given between and after ACgN treatments until 10 weeks; Group 10, 62.5 mg/kg MNU i.p. and continuous 1% ACgN treatment until 10 weeks; Group 11, 62.5 mg/kg MNU i.p. and continuous 4% ACgN treatment until 10 weeks. In the combined regimens, MNU was administered after the first 5 days of ACgN treatment. Assays of MT immunopositive crypts were carried out by two assessors (E.T.D. and H.B.). Working independently in Belfast and Cambridge, respectively, and were blind to treatment category. Observer variance was checked by each assessor re-scoring 90 randomly selected tissue sections. Study codes were broken and results compared on completion of the observer variance assessment.

Fig. 1. Treatment strategies for assessment of ACgN/MNU interactive effects upon MT immunopositive crypt indices. All ACgN treatments began on day 1. Timing and duration of 1 and 4% ACgN treatment are represented by light and dark shaded areas, respectively. In groups 8 and 9, each 7 day interval of ACgN exposure was followed by a 2 week ‘washout’ interval of drinking water only, without ACgN intake. MNU (62.5 mg/kg) was administered as a single i.p. injection, on day 5.

Animal welfare

Welfare considerations were in strict accordance with OECD guidelines (28,29,33).

Data analysis

Effects of ACgN alone on histological scores of mucosal injury were assessed by one-way between-group ANOVA. Effects of MNU alone or with ACgN upon 7-methylguanine and O6-methyldeoxyguanosine adduct formation were assessed using ANOVA for a factorial experimental design. Adduct levels were expressed per 106 nt and are described as the mean ± SD. MT immunopositive crypt indices were expressed per 105 total crypts. In MNU dose-response studies, MT immunopositive crypt data were log transformed and assessed by a probability plot of residuals to provide a normal distribution. Transformed data were analysed by one-way between-groups ANOVA. Formation of MT immunopositive patches was corrected for the confounding effect of physiological crypt fission. For each animal, the value for total number of MT immunopositive patches was divided by the frequency of MT immunopositive foci.

Serial animal weight data were available in individual mice. Incremental weight gain between study start and completion was calculated as a percentage increase over original weight for individual mice in each treatment group. Most study animals achieved 120% of their original weight by study completion. A Cox proportional hazards model was used to assess any time differences for initial achievement of 120% of original body weight between treatment groups. The weight index was calculated as the weight at study completion relative to the weight at study start, expressed as a percentage. Between-group differences in weight index were assessed by one-way ANOVA. Descriptive statistics applied to weight index were expressed as means ± SD. Group data were available for consumption of food and fluid, which were assessed in g/kg body wt or ml/kg body wt, respectively. Descriptive statistics were expressed as the mean. To achieve a normal distribution, MT immunopositive crypt indices from each MNU/ACgN treatment group were log transformed and assessed by a probability plot of residuals. Transformed data were analysed by ANOVA. Duncan post hoc tests were applied to assess differences between specific treatment regimens. Any correlation between colonic crypt mutational indices was investigated by Pearson’s product moment coefficient.
Table 1. Effects of continuous λCgN on crypt loss, inflammation and crypt hyperplasia/distortion

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Mice (n)</th>
<th>Crypt loss score</th>
<th>Inflammation score</th>
<th>Hyperplasia score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking water</td>
<td>5</td>
<td>0.2 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Continuous 1% λCgN</td>
<td>5</td>
<td>0.7 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Continuous 4% λCgN</td>
<td>5</td>
<td>1.6 ± 0.6</td>
<td>1.7 ± 0.6</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

Values shown are means ± SD. Continuous 1 or 4% λCgN induced greater hyperplasia ($P < 0.001$) and inflammation scores ($P = 0.05$ and 0.008, respectively) than the distilled drinking water control. 4% λCgN induced greater crypt loss than 1% λCgN ($P = 0.03$) or drinking water ($P = 0.002$) (ANOVA).

The SPSS for Windows (version 11) program was used for statistical analysis (SPSS Inc., Chicago, IL).

Results

Model validation studies: individual test agents given alone

Effects λCgN treatment on stool consistency, gross mucosal morphology and histology. Fifteen female Balb/c mice received drinking water only or continuous 1 or 4% λCgN in the drinking water for 10 weeks. All mice in the water-only treatment group had solid stools throughout the 10 week study interval. Stools became semi-soft after 32 days of continuous 1% λCgN. Mice receiving continuous 4% λCgN developed stools of this consistency within 1 week and soft semi-liquid stools after 11 days. Redness was found in the proximal 1/3 of the colon in all mice receiving 1 and 4% λCgN. In 3 mice of the 4% λCgN group, redness extended throughout the proximal 2/3 of the colon. No abnormalities were observed in the water-only treatment group. One-way ANOVA demonstrated significant between-group differences in crypt loss, mucosal inflammation and crypt hyperplasia/distortion scores (Table I and Figure 2).

MNU dose-response assay against DNA adduct formation and MT crypt immunopositivity. A mean ± SD of 1.32 ± 0.24 μmol DNA phosphate, corresponding to ~0.50 mg DNA, was isolated from each mouse colon. Administration of 62.5 or 125 mg/kg MNU induced dose-related formation of 7-methylguanine and $O^6$-methyldeoxyguanosine adducts in mouse colon, in excess of the distilled water or DMSO-treated control groups ($P < 0.001$; Table II). A further batch of 20 mice was treated with these regimens. Colonos were retrieved after 10 weeks for assay of MT immunopositive (mutant) crypt indices (Figure 3). One way ANOVA indicated significant between-group differences in the frequency of immunopositive foci ($P < 0.001$), total number of immunopositive crypts ($P < 0.001$) and numbers of patches of ≥ 2 immunopositive crypts ($P = 0.008$). MNU showed a dose-response effect upon adduct formation and total number of immunopositive crypts (Tables II and III).

Combined treatment studies

Effects of short-term λCgN treatment upon MNU-induced adduct formation in mouse colon. Colonos were retrieved 24 h after MNU treatment for assay of adduct levels. Levels of 7-methylguanine and $O^6$-methyldeoxyguanosine isolated from colonic DNA after administration of 1 or 4% λCgN with MNU were not significantly different from those of MNU alone (Table II).

Effects of treatment regimens on food, fluid intake and body weight. Eleven treatment groups of mice ($n = 90$) received λCgN alone or in combination with MNU (62.5 mg/kg i.p.) for assay of MT immunopositive crypt indices (Figure 1). Group values for fluid and food intake for these studies are shown (Table IV). The rate of weight gain was compared between groups by assessment of the time from the start of the study to initial gain of 120% of the original body weight. Values for mean weight index were also compared between treatment groups at study completion. Neither λCgN nor MNU treatment, whether administered alone or in combination, was associated with a significant delay of weight gain. No significant differences in group weight index were observed at study completion (Table IV).

Effects of treatment regimens upon colonic crypt stem cell mutation indices. Combined effects of λCgN and MNU on all MT immunopositive crypt indices were significantly greater than those of MNU alone or treatments lacking MNU. The Duncan post hoc test identified four homogeneous treatment subsets (A–D) which showed significant incremental differences in the frequency of MT immunopositive foci (mean ± SD/10⁴ crypts) as follows: Subset A (groups 1–4, no MNU, 0.8–1.1 ± 0.8–1.2/10⁴ crypts); Subset B (group 5, MNU alone), 18.7 ± 5/10⁴ crypts; Subset C (groups 6 and 7, MNU and one 7 day treatment with 1 or 4% λCgN), 29.4–30.4 ± 5.9–9.1/10⁴ crypts; Subset D (groups 8 and 10, MNU and three 7 day treatments with 1% λCgN or continuous 1% λCgN), 46.4–47.5 ± 10.6–14/10⁴ crypts. Groups 9 and 11 (MNU and three 7 day treatments with 4% λCgN or continuous 4% λCgN) had mean frequencies of MT immunopositive foci of 36.3–37.3 ± 9.6–9.7, which overlapped subsets C and D (Figure 4). Differences between subsets were statistically significant ($P < 0.001$).

One way between-groups ANOVA showed that combined λCgN/MNU dose regimens induced significantly greater total numbers of MT immunopositive crypts than MNU alone or treatments lacking MNU. The Duncan post hoc test identified the same four homogeneous treatment subsets (A–D) as previously shown in analysis of frequency of MT immunopositive foci. These subsets showed significant incremental differences in total number of MT immunopositive crypts. One-way ANOVA demonstrated significant between-group differences in MT immunopositive patch formation ($P < 0.001$) (Figure 5). Over 95% of patches involved only two contiguous MT immunopositive crypts. Mean patch frequency relative to frequency of MT immunopositive foci was 10.08 ± 0.4 for MNU alone versus 13.4 ± 0.6 for all combined λCgN/MNU treatment groups.

Assessment of inter-observer variance of MT immunohistochemistry was carried out. Mean ± SD frequencies of MT immunopositive foci for observers H.B. and E.T.D. were 9.9 ± 0.6 for MNU alone versus 95% of patches involved only two contiguous MT immunopositive crypts. Mean patch frequency relative to frequency of MT immunopositive foci was 10.08 ± 0.4 for MNU alone versus 13.4 ± 0.6 for all combined λCgN/MNU treatment groups.

Discussion

Establishing disease causality of any combination of dietary chemicals may be contingent upon knowledge of individual effects and elucidation of additive or interactive mechanisms. The present study assessed λCgN and MNU as model pro-oxidant and genotoxic chemicals alone and in combination against disease-relevant end-points in mouse colon. Undegraded λCgN was given at target organ toxic doses (34) within the limits of the OECD guidelines (29). We found that continuous oral λCgN treatment (1 or 4%) was associated with
laxation, redness of the proximal colonic mucosa and histological evidence of inflammatory mucosal injury. All parameters of inflammation, crypt loss and crypt hyperplasia were significantly increased in different colonic regions in λCgN-treated mice. Dose-dependent effects of MNU upon DNA adduct formation and MT immunopositive crypt indices were then tested in mouse colon.

Like other biomarkers based on mutagen-induced phenotype alteration (23,35,36) the assay of permanent crypt restricted MT immunopositivity is used to infer somatic mutations in

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Fig. 2. Histological assessment of λCgN pro-inflammatory effects. (a) Normal mouse colon (drinking water control). (b) Mucosal inflammation after continuous 1% λCgN treatment. Note focal inflammatory cell infiltration. (c) Crypt injury and loss. Note shortening and loss of crypts after continuous 1% λCgN treatment. (d) Crypt hyperplasia after continuous 1% λCgN treatment. Note long crypts and hyperchromatic epithelium and reduced goblet cell numbers. (e) Loss of surface epithelium after 4% λCgN treatment. Surface epithelium and crypts have been lost. One surviving hyperchromatic crypt is seen.
Values shown are means ± SD. 62.5 or 125 mg/kg MNU induced greater adduct formation than the water or DMSO controls (ANOVA, \( P < 0.001 \)).

Table II. Effects of MNU and λCgN alone and combined upon DNA adduct formation

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Mice (n)</th>
<th>7-Methylguanine adducts/10⁶ nt (mean ± SD)</th>
<th>8-methyldeoxyguanosine adducts/10⁶ nt (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water only</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>DMSO only</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1% λCgN only</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4% λCgN only</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>62.5 mg/kg MNU</td>
<td>5</td>
<td>132 ± 18</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>125 mg/kg MNU</td>
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<td>245 ± 32</td>
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</tr>
<tr>
<td>62.5 mg/kg λCgN</td>
<td>5</td>
<td>139 ± 16</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>MNU + 1% λCgN</td>
<td>5</td>
<td>146 ± 18</td>
<td>19 ± 3</td>
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</tbody>
</table>

Values shown are means ± SD. 62.5 or 125 mg/kg MNU induced greater adduct formation than the water or DMSO controls (ANOVA, \( P < 0.001 \)).

Table III. Effects of MNU dose upon MT immunopositive colonic crypt mutations

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Mice (n)</th>
<th>Total crypt no.</th>
<th>Mutation frequency</th>
<th>Patches ≥2 mutant crypts</th>
<th>Total mutation load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water only</td>
<td>5</td>
<td>35904 ± 7886</td>
<td>0.81 ± 0.83</td>
<td>0.25 ± 0.26</td>
<td>1.07 ± 0.98</td>
</tr>
<tr>
<td>DMSO only</td>
<td>5</td>
<td>33982 ± 5725</td>
<td>1.4 ± 1.2</td>
<td>0.21 ± 0.21</td>
<td>1.7 ± 1.4</td>
</tr>
<tr>
<td>62.5 mg/kg MNU</td>
<td>5</td>
<td>29886 ± 4260</td>
<td>16.6 ± 8.5</td>
<td>0.85 ± 0.33</td>
<td>17.48 ± 8.8</td>
</tr>
<tr>
<td>125 mg/kg MNU</td>
<td>5</td>
<td>35067 ± 6565</td>
<td>31.7 ± 15.0</td>
<td>3.76 ± 3.1</td>
<td>35.2 ± 18.5</td>
</tr>
</tbody>
</table>

Values shown are means ± SD. 62.5 or 125 mg/kg MNU induced a greater mutation frequency or total mutation load than the water or DMSO control (ANOVA, \( P < 0.001 \)). Mutant patch formation was greater with 125 mg/kg MNU versus control.

Table IV. Combined studies: effects of treatment on group fluid, food intake and weight index at study completion

<table>
<thead>
<tr>
<th>Treatment regimens</th>
<th>Mice (n)</th>
<th>Group fluid intake (ml/g body wt)</th>
<th>Group food intake (g/g body wt)</th>
<th>Weight index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Water only</td>
<td>5</td>
<td>0.116</td>
<td>0.119</td>
<td>121.80 ± 4.8</td>
</tr>
<tr>
<td>2. DMSO only</td>
<td>5</td>
<td>0.122</td>
<td>0.134</td>
<td>122.55 ± 5.8</td>
</tr>
<tr>
<td>3. 1% Continuous λCgN</td>
<td>5</td>
<td>0.112</td>
<td>0.134</td>
<td>121.48 ± 7.9</td>
</tr>
<tr>
<td>4. 4% Continuous λCgN</td>
<td>5</td>
<td>0.088</td>
<td>0.138</td>
<td>120.18 ± 4.1</td>
</tr>
<tr>
<td>5. 62.5 mg/kg MNU only</td>
<td>10</td>
<td>0.106</td>
<td>0.112</td>
<td>119.77 ± 4.6</td>
</tr>
<tr>
<td>6. MNU + 1 × 7 day treatment cycle of 1% λCgN</td>
<td>10</td>
<td>0.098</td>
<td>0.115</td>
<td>122.45 ± 4.2</td>
</tr>
<tr>
<td>7. MNU + 1 × 7 day treatment cycle of 4% λCgN</td>
<td>10</td>
<td>0.098</td>
<td>0.098</td>
<td>124.49 ± 4.9</td>
</tr>
<tr>
<td>8. MNU + 3 × 7 day treatment cycles of 1% λCgN</td>
<td>10</td>
<td>0.107</td>
<td>0.109</td>
<td>122.49 ± 13.3</td>
</tr>
<tr>
<td>9. MNU + 3 × 7 day treatment cycles of 4% λCgN</td>
<td>10</td>
<td>0.107</td>
<td>0.114</td>
<td>123.80 ± 3.8</td>
</tr>
<tr>
<td>10. MNU + continuous 1% λCgN</td>
<td>10</td>
<td>0.109</td>
<td>0.109</td>
<td>123.87 ± 9.3</td>
</tr>
<tr>
<td>11. MNU + continuous 4% λCgN</td>
<td>10</td>
<td>0.089</td>
<td>0.111</td>
<td>120.25 ± 8.4</td>
</tr>
</tbody>
</table>

Group values only were available for fluid or food intake. Individual animal weights were available. The weight index was calculated as the weight at study completion relative to weight at study start, expressed as a percentage. Values shown are means of group fluid or food intake and means ± SD for weight index. No between-group differences in weight index were found (ANOVA).

On this basis, stable, crypt restricted immunopositivity for MT is considered to result from a mutation affecting expression of the MT gene in a colonic stem cell (26). After such an event, clonal stabilization, elimination of any confounding MT immunopositive transitory cells by homeostatic turnover and permanent re-population of the crypt by MT immunopositive stem cell progeny occurs over an interval of 6 weeks (25). These temporal events are similar to those observed after G6PD mutation in crypt stem cells (37). To accommodate these events, the study design incorporated a minimum interval of 10 weeks between MNU treatment and MT assay. In this study MNU treatment induced dose-dependent effects upon adduct formation and MT crypt immunopositivity.
A principal objective of this study was to investigate any additive or interactive effects of a non-genotoxic diet-related chemical upon NOC-induced mutational events. Human exposure to carrageenan is variable (38), occurring by consumption of foods like processed meat (39), bread (40), sauces (41) and desserts (14). The present study modelled this consumption variance by regimens of different λCgN dose and continuity of exposure, alone or in combination with a single treatment with 62.5 mg/kg MNU. Treatment with continuous λCgN alone was pro-inflammatory in this study but had no demonstrable effects on MT immunopositive crypt indices. Hence, any oxidative DNA modifications due to λCgN treatment were not converted to MT immunopositive crypt foci. Short-term treatment with 1 or 4% λCgN had no effect upon MT immunopositive crypt indices, effects with MNU were considered interactive rather than additive (42).

Interactions between genotoxic and non-genotoxic agents may affect physico-chemical properties of mutagenic substances, efficacy of DNA repair or other mechanisms (43). Carrageenans enhance tissue expression of nitric oxide (9), which effectively inhibits O6-alkylguanine-DNA alkyltransferase, the DNA repair enzyme responsible for removal of the pro-mutagenic adduct O6-methyldeoxyguanosine (44). This mechanism could promote somatic mutation (45). Increased cell proliferation induced by carrageenan (46) could also enhance mutability (47). In a state of sustained λCgN-induced inflammation, the pro-inflammatory cytokine MIF could inhibit p53-mediated elimination of cells bearing irretrievable DNA damage (48), thus enhancing mutation. However, further work is required to determine the precise molecular mechanisms of the λCgN/MNU interaction.

Non-genotoxic chemicals may promote clonal expansion by crypt fission, leading to development of ‘patches’ of two or more MT immunopositive crypt foci. Treatment groups: 1, water only; 2, DMSO only; 3, continuous 1% λCgN only; 4, continuous 4% λCgN only; 5, 62.5 mg/kg MNU only; 6, MNU + 1 × 7 day cycle of 1% λCgN; 7, MNU + 1 × 7 day cycle of 4% λCgN; 8, MNU + 3 × 7 day cycles of 1% λCgN; 9, MNU + 3 × 7 day cycles of 4% λCgN; 10, MNU + continuous 1% λCgN; 11, MNU + continuous 4% λCgN. Error bars denote ±1.0 SD. Significant differences have been identified between four homogeneous subsets, namely: groups 1–4 (no MNU); group 5 (62.5 mg/kg MNU alone); groups 6 and 7 (62.5 mg/kg MNU + 1 × 7 day exposure to 1 or 4% λCgN); groups 8 and 10 (62.5 mg/kg MNU + 3 × 7 day exposure of 1% λCgN or continuous 1% λCgN). Differences between each of the subsets are statistically significant (P < 0.001). Groups 9 and 11 (62.5 mg/kg MNU + 3 × 7 day treatments of 4% λCgN or continuous 4% λCgN) overlapped the third and fourth subsets.

OECD recommended limits (28,29). It appears unlikely that indirect effects of treatment combinations or inter-observer variance of MT immunohistochemistry, which was always <10%, could account for observed outcomes. Effects of combined λCgN/MNU regimes upon crypt stem cell mutational frequency appeared to be less with 4% than with 1% λCgN, although differences were not statistically significant. These data could result from a dose saturation effect for λCgN, possibly as a result of excessive tissue injury, cell death, inhibition of healing or other mechanism. Since λCgN treatment alone was ineffective against MT immunopositive crypt indices, effects with MNU were considered interactive rather than additive (42).
more adjacent mutant crypts (23). After mutagen treatment, physiological crypt fission promotes development of mutant patches in proportion to the mutation frequency (37). The combined $\lambda$CgN/MNU regimens in the present study, induced significantly greater MT immunopositive patch formation than MNU alone. To assess any promotional effects of $\lambda$CgN upon MT immunopositive patch formation in excess of that due to physiological fission, we calculated patch formation as a percentage of the frequency of MT immunopositive foci. This parameter was considered representative of mutation frequency. Values were higher with combined $\lambda$CgN/MNU regimens than with MNU alone. These data together with the morphological observations strongly suggest that $\lambda$CgN induces crypt fission, resulting in progression of a higher proportion of single MT immunopositive crypt mutations to patches. This mechanism may contribute to the increased total number of immunopositive crypts associated with combined $\lambda$CgN/MNU regimens.

Inconsistent opinions pertaining to health effects of carrageenan (14,49) underline the need for additional data. The present study shows that undegraded $\lambda$CgN enhanced the effect of a synthetic N-nitroso compound upon MT crypt immunopositivity in mouse colon. Synergy of toxic effects in short-term, high dose studies, however, does not necessarily reflect interactions at lower concentrations, commensurate with human exposures. Hence, accurate human intake data pertaining to carrageenans and N-nitroso compounds are required. In addition, further detailed studies are indicated to determine any $\lambda$CgN interaction threshold, the magnitude of interactive effects, dose–response effects and saturation levels and their relevance to human exposure.

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References


