Metallothionein crypt-restricted immunopositivity indices (MTCRII) correlate with aberrant crypt foci (ACF) in mouse colon

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Keywords: stem cell; mutation; mixture

Humans are exposed to mixtures of genotoxic and nongenotoxic environmental chemicals that may be linked to cancer (Burkart and Jung, 1998; Minamoto et al, 1999). Robust biomarkers of somatic stem cell mutation and mutant clonal expansion may provide cancer surrogates that are useful for risk assessment. Acquired mutation of a selectable endogenous reporter gene like glucose-6-phosphate dehydrogenase (G6PD) within a colonic crypt stem cell; mutation; mixture

MATERIALS AND METHODS

Chemicals, reagents, animals and treatment regimens were as described previously (Donnelly et al, 2004). Anti-MT primary antibody (mouse anti-horse monoclonal E9, isotype: IgG1) and peroxidase-conjugated rabbit anti-mouse immunoglobulins
were obtained from DAKO Ltd, Ely, Cambridgeshire, UK (Dako M0639 and P0161 respectively), as described previously (Jasani et al, 1998; Cook et al, 2000). Methylene blue was obtained from BDH Chemicals Ltd, Poole, Dorset, UK (BDH 34048).

Animals and treatment regimes
Female adult Balb/c mice, aged 6–8 weeks, were obtained from Harlan UK Ltd, Bicester, Oxon, UK, divided into groups of five or 10, ear-punched and placed in coded stainless-steel wire cages, maintained and fed as outlined previously (Donnelly et al, 2004). Individual animal weights and group fluid and AIN-76 diet consumption were assessed daily, during weekdays.

Treatment groups
In all, 90 female adult Balb/c mice aged 6–8 weeks were divided into 11 groups of five or 10 that received no MNU, MNU (62.5 mg kg−1 dissolved in dimethylsulphoxide (DMSO)) alone or in combination with 1 or 4% 3CgN as follows:

- **Group 1 (n = 5)**: Drinking water only for 20 weeks (water only control).
- **Group 2 (n = 5)**: Single intraperitoneal (i.p.) injection of MNU (DMSO), then drinking water for 20 weeks (vehicle control).
- **Group 3 (n = 5)**: Continuous 1% 3CgN only for 20 weeks.
- **Group 4 (n = 5)**: Continuous 4% 3CgN only for 20 weeks.
- **Group 5 (n = 10)**: MNU 62.5 mg kg−1 i.p., then drinking water only for 20 weeks.
- **Group 6 (n = 10)**: MNU 62.5 mg kg−1 i.p. and 1% 3CgN for 7 days during week 1, then drinking water until 20 weeks.
- **Group 7 (n = 10)**: MNU 62.5 mg kg−1 i.p. and 4% 3CgN for 7 days during week 1, then drinking water until 20 weeks.
- **Group 8 (n = 10)**: MNU 62.5 mg kg−1 i.p. and three 7-day treatments of 1% 3CgN during weeks 1, 4 and 7. Drinking water was given between and after 3CgN treatments until 20 weeks.
- **Group 9 (n = 10)**: MNU 62.5 mg kg−1 i.p. and three 7-day treatments of 4% 3CgN during weeks 1, 4 and 7. Drinking water was given between and after 3CgN treatments until 20 weeks.
- **Group 10 (n = 10)**: MNU 62.5 mg kg−1 i.p. and continuous 1% 3CgN treatment until 20 weeks.
- **Group 11 (n = 10)**: MNU 62.5 mg kg−1 i.p. and continuous 4% 3CgN treatment until 20 weeks.

In combined regimens, MNU was administered after the first 5 days of 3CgN treatment.

Welfare considerations and weight index
Animal welfare considerations were strictly in accordance with OECD guidelines (OECD, 2002). Animals were weighed daily and weight index was calculated as the ratio at study completion relative to weight at study start. Values for mean weight index were compared between treatment groups, at study completion.

Assay of ACF
All assays of ACF were blinded to treatment and carried out after colonic retrieval at 20 weeks after the initiation of treatment. Colons were carefully pinned flat on a cork mat, painted with 0.1% methylene blue and left at room temperature for 10 min. Assay of ACF was performed using a dissecting microscope at ×40 magnification and the following parameters were recorded:

- (i) ACF number: Assessed as the total number of ACF per colon or per 104 colonic crypts.
- (ii) ACF size (crypt multiplicity): Crypt multiplicity was determined as the number of aberrant crypts per ACF.

Colons were then ‘Swiss-rolled’ on the cork mat, with the ileoecal junction at the centre of the roll, fixed in neutral formal saline for 48 h, and embedded in paraffin wax blocks.

Assay of MTCRII
All assays of MTCRII were blinded to treatment and carried out 20 weeks after the initiation of treatment. MTCRII were assayed as described previously (Donnelly et al, 2004). Briefly, paraffin-embedded sections (4 μm thickness) were cut at 10 levels (L1–L10), 100 μm apart through the ‘Swiss-rolled’ colon. One section from each level was stained using a standard indirect immunoperoxidase technique for MT, while endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol. Slides were incubated with an anti-MT primary antibody (E9, isotype: IgG1; 100 μl per slide) (DAKO Ltd, Ely, Cambridgeshire, UK). The secondary antibody used was horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako catalogue no. P0161). 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 IgG subclass (1:1000) (IgG1; Dako catalogue product code X0931). These were consistently negative. Positive control sections included mouse colon previously treated with N-ethyl-N-nitrosourea (ENU; 250 mg kg−1), which induces MT-immunopositive crypts (Cook et al, 2000). Sections were washed and 100 μl of the chromogen 3,3′-diaminobenzidine tetrahydrochloride was added before counter-staining with Harris haematoxylin. The frequency and size of MT-immunopositive foci as well as total number of MT-immunopositive crypts were assessed as follows:

- (i) MT-immunopositive single crypts or patches of ≥2 contiguous MT-immunopositive crypts were recognised by their dark brown stain against a haematoxylin background and were assessed in transverse or longitudinal section through the crypt lumen. Each single or contiguous patch of ≥2 MT-immunopositive crypts was considered to represent a single mutant focus.
- (ii) The size of each MT-immunopositive patch was assessed by the number of contiguous MT-immunopositive crypts within the patch. Patches were recorded as doubles, triples or greater (n = 2, 3, etc., MT-immunopositive crypts).
- (iii) The total number of MT-immunopositive crypts per mouse colon was determined by the sum of single immunopositive and all immunopositives within patches.

Endpoints of the (i) frequency of MT-immunopositive foci (ii) number of MT-immunopositive patches and (iii) the total number of MT-immunopositive crypts were expressed as the number per 104 total crypts, in mouse colon.

Data analysis
Serial weight data were available in individual mice. The weight index was calculated as the weight at study completion relative to weight at study start, expressed as a percentage. Between-group differences of weight index were assessed by one-way ANOVA. Descriptive statistics applied to weight index were expressed as mean ± standard deviation (mean ± s.d.). Group data were available for consumption of food and fluid, which were assessed in grams or ml per kg body weight, respectively. Descriptive statistics
were expressed as mean±s.d. To achieve a normal distribution, MTCRII and ACF data from each treatment group were log-transformed to ensure a normal distribution and assessed by a probability plot of residuals. Transformed data were analysed by univariate ANOVA. Duncan post hoc tests were applied to assess differences between specific treatment regimens. Differences of MT-immunopositive patch formation between MNU alone and all combinations of λCgN/MNU were assessed by Student’s t-test. Correlations between MTCRII and ACF data were investigated by Pearson’s product moment coefficient. SPSS for Windows (version 11) was used for statistical analysis (SPSS Inc., Chicago, Il, USA).

**RESULTS**

**Food, fluid intake and body weight**

In all, 11 treatment groups of mice (n = 90 total) received water- or vehicle-only controls, high- or low-dose λCgN alone or in combination with MNU (62.5 mg kg⁻¹ i.p.). λCgN was given in single or recurrent short- or long-term patterns of exposure. Group values for fluid, food intake and weight index are shown in Table 1. No significant between-group differences of weight index were observed at study completion (Table 1).

| Treatment regimes | Mice (n) | Group fluid intake (mL g⁻¹ body weight) | Group food intake (g g⁻¹ body weight) | Weight index |
|-------------------|---------|----------------------------------------|--------------------------------------|-------------|
| 1. Control (water) only | 5       | 0.165                                  | 0.184                                | 132.72±2.2  |
| 2. DMSO vehicle only | 5       | 0.163                                  | 0.192                                | 133.71±2.5  |
| 3. Continuous 1% λCgN | 5       | 0.172                                  | 0.174                                | 126.51±3.4  |
| 4. Continuous 4% λCgN | 5       | 0.113                                  | 0.184                                | 132.09±4.7  |
| 5. MNU (62.5 mg kg⁻¹) | 10      | 0.152                                  | 0.161                                | 126.58±6.5  |
| 6. MNU+1 x 7 day 1% λCgN | 10     | 0.151                                  | 0.170                                | 130.49±6.9  |
| 7. MNU+1 x 7 day 4% λCgN | 10     | 0.149                                  | 0.165                                | 126.43±5.4  |
| 8. MNU+3 x 7 day 1% λCgN | 10     | 0.149                                  | 0.167                                | 133.29±6.9  |
| 9. MNU+3 x 7 day 4% λCgN | 10     | 0.122                                  | 0.165                                | 129.95±3.9  |
| 10. MNU+continuous 1% λCgN | 10     | 0.137                                  | 0.164                                | 130.22±4.6  |
| 11. MNU+continuous 4% λCgN | 10     | 0.114                                  | 0.165                                | 125.41±6.2  |

DMSO = dimethylsulphoxide; λCgN = lambda carrageenan; MNU = N-methyl-N-nitrosourea.

**Table 1** Effects of 20-week treatments on group fluid and food consumption and weight index

**Table 2** Treatment effects upon MTCRII

| Treatment | Mice (n) | Total MT-immunopositive crypt number | Patches ≥ 2 MT-immunopositive crypts | Frequency of MT-immunopositive foci |
|-----------|---------|-------------------------------------|--------------------------------------|-------------------------------------|
| 1. Water only | 5       | 36.43±2.053                         | 1.13±0.04                           | 0.10±0.06                           | 1.13±0.33                          |
| 2. DMSO only | 5       | 33.29±2.888                         | 0.97±0.29                           | 0                                   | 0.97±0.29                          |
| 3. Continuous 1% λCgN | 5       | 37.02±2.091                         | 0.88±0.32                           | 0                                   | 0.88±0.32                          |
| 4. Continuous 4% λCgN | 5       | 38.67±1.149                         | 1.3±0.32                            | 1                                  | 1.3±0.32                           |
| 5. MNU only (62.5 mg kg⁻¹) | 10      | 29.75±1.925                         | 37.66±2.93                          | 5.54±0.91                          | 31.73±2.66                          |
| 6. MNU+1 x 7-day cycle 1% λCgN | 10     | 27.71±1.715                         | 46.54±5.53                          | 6.76±1.10                          | 38.60±5.18                          |
| 7. MNU+1 x 7-day cycle 4% λCgN | 10     | 32.53±1.543                         | 52.31±1.482                         | 9.61±0.78                          | 39.39±4.72                          |
| 8. MNU+3 x 7-day cycles 1% λCgN | 10     | 30.65±1.201                         | 59.85±6.91                          | 8.27±1.56                          | 51.46±6.81                          |
| 9. MNU+3 x 7-day cycles 4% λCgN | 10     | 30.64±1.316                         | 52.47±7.80                          | 9.62±0.67                          | 43.79±5.89                          |
| 10. MNU+continuous 1% λCgN | 10     | 31.37±1.832                         | 47.86±5.96                          | 8.00±1.14                          | 38.14±5.11                          |
| 11. MNU+continuous 4% λCgN | 10     | 23.45±1.007                         | 56.23±5.41                          | 5.82±0.69                          | 49.98±5.09                          |

MTCRII = metallothionein crypt-restricted immunopositivity indices; MT = metallothionein; DMSO = dimethylsulphoxide; λCgN = lambda carrageenan; MNU = N-methyl-N-nitrosourea.

**Treatment effects upon MTCRII**

Group values for MTCRII, including total number of MT-immunopositive crypts, MT-immunopositive patch formation and frequency of MT-immunopositive foci, are shown in Table 2. The total number of MT-immunopositive crypts was increased by >25-fold in excess of that of vehicle alone, by MNU (62.5 mg kg⁻¹) treatment, but was unaffected by λCgN treatment alone. Data analysis by one-way between-group ANOVA with the Duncan post hoc test allowed division of results into statistically different subsets. Combined λCgN/MNU regimens induced significantly greater total number of MT-immunopositive crypts compared to MNU alone or treatments lacking MNU (P<0.01; Table 2). Significant incremental differences were observed between treatment subsets (A–C), where A represents treatment groups 1–4, B represents groups 5 and 6 and C represents groups 8 and 11. Treatment groups 7, 9 and 10 overlapped subsets B and C (Figure 1A).

Significant between-group differences in the frequency of patches of ≥2 contiguous MT-immunopositive crypts were also observed (P<0.05; ANOVA). Significant incremental differences were observed between three treatment subsets (A–C), where A represents treatment groups 1–4, B represents group 5 and C represents groups 7 and 9. Treatment groups 6, 8, 10 and 11 overlapped subsets B and C (Figure 1B). Over 95% of mutant patches involved only two contiguous mutant crypts. The frequency of large MT-immunopositive patches (≥3 contiguous immunopositive crypts) was 0.38±0.05 per 10⁴ total crypts for MNU alone (group 5) vs 1.12±0.13 per 10⁴ total crypts for all λCgN/MNU treatment groups (P=0.002). All patches of ≥4 MT-immunopositive crypts were observed in combined λCgN/MNU treatment groups.

**Effect of treatment regimes on ACF frequency**

The administration of MNU led to a significant increase in ACF numbers by about 10-fold in excess of that of DMSO vehicle alone. ACF data were expressed either as a number per 10⁴ total colonic crypts (Figure 2A) or per mouse colon (Table 3). λCgN treatment alone led to a small significant increase in ACF size, in terms of crypt multiplicity but had no significant effect on ACF number. Combined λCgN/MNU regimens significantly increased ACF number and size (P<0.001; ANOVA). Post hoc analysis demonstrated significant incremental differences in ACF number between five homogeneous treatment subsets (A–E), where A represents treatment groups 1–4, B represents treatment group 5, C represents groups 6 and 7, D represents group 8 and E represents groups 9 and 11. Group 10 overlapped subsets C and D (Figure 2A; Table 3). Significant effects of treatment on ACF size were also observed (P<0.01; Figure 2B) with incremental differences in...
Correlations between MTCRII and ACF

Assessments of MTCRII and ACF were conducted in all treatment groups (1–11). Linear correlations were observed between total MT-immunopositive crypt number per $10^4$ crypts and ACF number per $10^4$ crypts ($r = 0.732; P < 0.01$) (Figure 3A) and ACF size, in terms of the number of aberrant crypts per focus ($r = 0.84; P < 0.01$) (Figure 3B).

DISCUSSION

Colonic tumorigenesis involves acquisition of mutations or heritable epigenetic events, affecting growth control or differentiation genes within crypt stem cells, progression to premalignant stages including ACF (Bird and Good, 2000) and ultimate invasive
carcinoma. Since these events are stochastic, a higher stem cell mutation rate may accelerate distinct stages of this process (Herrero-Jimenez et al, 1998). Robust biomarkers of stem cell mutation may thus provide useful surrogates of tumorigenesis. Metallothionein crypt-restricted immunopositivity indices provide a stem cell mutation marker that is initiated by mutagen exposures, yet mimics sporadic tumorigenesis because it occurs in widely scattered single crypts or foci throughout the otherwise normal colon (Cook et al, 2000).

Since the relationship of MT crypt-restricted immunopositivity to tumorigenesis was unclear, we assessed the relationship between MTCRII and ACF frequency, in mice treated by λCgN and MNU.

The present study uses a similar combinatorial design, involving a single MNU treatment (62.5 mg kg⁻¹) together with single, repeated or continuous exposures to low- (1%) or high- (4%) dose λCgN, to that of our previous study (Donnelly et al, 2004). In the present study however, follow-up and continuous λCgN treatment were continued for longer term (20 weeks). The present study supports our earlier work and shows that λCgN alone does not significantly affect MTCRII, but enhances MNU effects upon this end point (Donnelly et al, 2004). However, sequential or prolonged λCgN exposure to 20 weeks was associated with the development of larger MT-immunopositive (mutant) patches than observed at 10 weeks, in our previous study.

Hence, prolonged λCgN exposure may have cumulative effects upon mutant patch size. These effects could be related to λCgN-induced tissue injury in mouse colon (Donnelly et al, 2004), fission of immunopositive crypts and formation or enlargement of immunopositive patches, during continual or repeated regenerative healing.

While biomarkers of rate-limiting steps of tumorigenesis are informative, validation against tumour-associated end points is important. Aberrant crypt foci comprise a contiguous collection of crypts that have thickened epithelia, altered luminal openings and are clearly circumscribed from adjacent normal crypts (Bird, 1987). Gene mutations that are commonly observed in colon cancers including K-ras and APC are also observed in a proportion of ACF (Pretlow et al, 1993; Smith et al, 1994). Aberrant crypt foci are thus considered to represent early-stage colorectal tumorigenesis (Bird, 1987; Tudek et al, 1989; Takayama et al, 1998; Bird and Good, 2000), although large or persistent ACF may have greater cancer risk (Papanikolaou et al, 2000). The present study has shown that MTCRII may reflect combined effects of chemicals within a mixture, are induced in sufficient numbers to provide statistical power from relatively small animal samples and correlate with ACF formation at 20 weeks after the initiation of treatment. MTCRII may thus provide the basis for an intermediate risk assessment model for diet- or lifestyle-related genotoxic/nongenotoxic chemical combinations, relevant to colonic health.

**ACKNOWLEDGEMENTS**

This study was funded by Research Contract T01018 from the Food Standards Agency, which is gratefully acknowledged.
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British Journal of Cancer (2005) 92(12), 2160 – 2165