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# Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay

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**Human faecal waters from 35 healthy non-smoking volunteers (23 from England and 12 from Sweden) consuming their habitual diet were screened for genotoxicity by the single-cell gel electrophoresis (comet) assay using a human colon adenocarcinoma cell line (CACO-2) as the target. Hydrogen peroxide induced DNA damage was categorized as low, intermediate or high for tail moments greater than 5, 17 and 32, respectively: 11 samples were highly genotoxic, four were intermediate, one was low and 19 showed no activity. Endonuclease III treatment significantly increased DNA damage for all except the non-genotoxic faecal waters, suggesting that faecal water genotoxicity may be due, at least in part, to oxidative damage. Faecal water cytotoxicity has previously been attributed to the bile and fatty acid content. In the comet assay no DNA damage was induced by deoxycholate or lithocholate at normal physiological concentrations, suggesting that the genotoxicity of faecal water was due to other substances. Both bile acids induced DNA damage above 300 μM, levels often found in patients with colonic polyps and there was a significant increase in genotoxicity after endonuclease III treatment indicative of oxidative DNA damage.**

## Introduction

Of all environmental factors which appear to play a role in the aetiology of colon cancer, diet seems to be the most important (1). Dietary constituents or the products of their digestion or metabolism may provoke neoplastic change (2), and oxidative DNA damage induced by faecal compounds and/or secondary bile acids is a suspected mechanism for tumour formation in the colon (3). It can be hypothesized, therefore, that colon cancer may result from direct-acting carcinogens in food, or by the generation of genotoxic species within the colon by host-mediated or gut microflora-associated metabolism.

In recent years, interest has focused on the aqueous phase of human faeces (faecal water) by investigators studying the mechanisms underlying the dietary aetiology of colon cancer. This has been motivated by several studies which demonstrated that components of this faecal fraction are more efficient in altering the growth characteristics of colonocytes than components of the solid phase (4–6).

It has been postulated that colon carcinogens are only present at very low levels. Since carcinogenicity assays are, in general, very insensitive, requiring high doses of moderately potent carcinogens to produce convincing results, methods for detecting biological activity characteristic of carcinogens (DNA damage and mutagenicity) have been employed (7). Genotoxic activity in human faeces, but not in faecal water, has previously been detected using a variety of mutagenic tests; the Ames test, bacterial fluctuation tests, differential survival test, SOS Chromotest (7) and short-term tests using induction of chromosomal anomalies in cultured mammalian cell lines (8). However, DNA damage in colonic cells has not previously been demonstrated. Studies using induction of chromosomal anomalies in cultured mammalian cells with faeces have been performed (8), although cells were not derived from human colon or colonic tumours and more appropriate models for the generation of genotoxicity data with relevance to the human colon are required. Results from previous tests have provided conflicting estimates for the numbers of people excreting genotoxic faeces, with the Ames test indicating that most people excrete faecal mutagens whilst the fluctuation test indicates a level of between 20 and 30% for populations on a typical Western diet (7).

The majority of the test systems outlined above evaluated the mutagenic effects of faecal extracts on bacterial DNA, and there is little data from studies on mammalian DNA and none from experiments involving human colon cells. In the current study the single-cell gel-electrophoresis (SCGE*) or comet assay (9) was used to assess the genotoxic effects of faecal water on CACO-2 cells; a human colonic adenocarcinoma cell line. The comet assay is a sensitive and rapid technique able to detect DNA strand breaks and alkali labile sites in individual cells (10,11) and has previously been used to investigate genotoxicity both *in vitro* (12) and *in vivo* (13,14).

This study also addressed whether the DNA damage was induced by an oxidative mechanism, and evaluated possible candidate components in the faecal water that may have caused the damage. By addition to the comet slides of *Escherichia coli* endonuclease III (EndIII), an enzyme which nicks DNA at sites of oxidized pyrimidines and which is specific for oxidative base damage (15), the comet assay is able to indicate whether oxidized pyrimidines are present following incubation with faecal water or bile acid.

Whilst there is little evidence linking bile acid derivatives with direct DNA damage *in vitro* or *in vivo*, bile acids have been implicated in colon carcinogenesis: At physiological concentrations, secondary bile acids are known to be cytotoxic to human colon tumour cell lines including HT29, HCT 116 and $T_{84}$ using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (16) and erythrocyte-lysis assay (6), whilst two primary bile acids (cholic and chenodeoxycholic acid) have been shown to have a promoting effect

***Abbreviations:** SCGE, single cell gel electrophoresis; EndIII, *Escherichia coli* endonuclease III; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; LCA, lithocholic acid; DCA, deoxycholic acid; EMEM, Eagle's Minimum Essential Medium; RPMI, Roswell Park Memorial Institute; BSA, bovine serum albumin; LMA, low melting-point agarose.

in colorectal cancer in germ-free and conventional F344 rats which had previously received intra-rectal 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) (17). In addition, the excretion of secondary bile compounds in the colon are likely to be increased when high fat, low calcium and low fibre diets are consumed (18); diets which are known increase the risk of colon cancer.

## Materials and methods

*Materials*

CACO-2 cells were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. Lithocholic acid (LCA), deoxycholic acid (DCA) and its sodium salt (Na-DCA) were purchased from Sigma Chemical Company (Poole, Dorset). Stock solutions (100mM) of LCA, DCA-free acid and Na-DCA were prepared in ethanol then diluted in RPMI 1640 medium before use in the comet assay; the maximum final concentration of ethanol was 1%.

Eagle's Minimum Essential Medium (EMEM) and Roswell Park Memeorial Institute (RPMI) 1640 medium containing 20 mM glutamine were purchased from Gibco Life Technologies Ltd., Paisley, Scotland.

Freeze-dried endonuclease III was kindly provided by Dr Andrew Collins from the Rowett Research Institute, Aberdeen, Scotland. The enzyme was reconstituted with 100 µl of enzyme reaction buffer [40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA); pH 8 with KOH], and 10 µl aliquots stored frozen at –80°C. Before use, 990 µl of enzyme reaction buffer was added to the 10 µl aliquot.

*Faecal water preparation*

The study was conducted with the approval of an independent Ethics Committee and with the informed consent of the participants.

Initially, four volunteers were asked to provide two faecal samples on different days to assess the optimum concentration of faecal water to use in the comet assay. Genotoxic activity was assessed using faecal water at concentrations of 4 or 10% in the incubation.

For the main study, healthy, non-smoking male and female volunteers (23 from the United Kingdom and 12 from Sweden), aged 17–57, with no history of gastro-intestinal disease or antibiotic use in the previous 3 months, were asked to provide complete faecal samples (the total faeces of one passage). Faecal samples were collected and kept on ice before processing. Samples were homogenized in a stomacher for 2 min and centrifuged at 50 000 ***g*** for 2 h at 20°C. The supernatant (faecal water) was carefully decanted into 1.5 ml Eppendorf tubes which were stored frozen (–20°C) until analysis. Sample weights and supernatant volumes were recorded.

*Comet assay*

CACO-2 cells were cultured as monolayers in EMEM (containing 20% foetal calf serum, 10 µg/µl bovine insulin, 20 mM L-glutamine and 0.6% penicillin-streptomycin) for 7 days at 37°C. For use in genotoxicity assays with faecal water samples or bile acids, cells were liberated after a 5 min incubation with 0.25% trypsin-EDTA at 37°C and resuspended in RPMI 1640 medium.

The methodology described previously (19) was followed with small modifications. Briefly, CACO-2 cells were incubated in Eppendorf tubes for 30 min at 37°C with either faecal water samples (50 + 450 µl cell suspension) or bile acid. The bile acids LCA and DCA were dissolved in ethanol then diluted with RPMI to give a range of concentrations from 100 to 1000 µM final concentration (the final concentration of ethanol was 1%). Cell viability was assessed before and after the incubation by trypan blue exclusion. Positive controls (25–100 µM $H_2O_2$) and negative controls (saline for faecal waters; 1% ethanol in RPMI for bile acids) were included with each experiment.

Cells were centrifuged at 100 ***g*** for 3 min, the supernatant discarded and cells resuspended in 75 µl of 0.7% low melting point agarose (LMA; Gibco BRL, Life Technologies Inc., Gaithersburg, USA) made in phosphate buffered saline (PBS) and maintained at 37°C for embedding on microscope slides. Cells were lysed (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM TRIS; containing 1% Triton X-100 and 10% dimethylsulphoxide) for 1 h at 4°C. The DNA was allowed to unwind in electrophoresis buffer (1 mM $Na_2EDTA$, 300 mM NaOH, pH 13.5) at 4°C for 20 min before electrophoresis for 20 min at 26 V, 300 mA (0.037 V/cm$^2$).

After electrophoresis, slides were washed in neutralization buffer (0.4 M tris, pH 7.5) then stained with 85 µl of ethidium bromide (2 µg/ml) and stored in a moist atmosphere at 4°C for no longer than 48 h before scoring. Slides were analysed at 600× magnification using a fluorescence microscope (Zeiss-Photomicroscope II). One-hundred randomly selected cells from each slide were measured and tail moments recorded using KOMET 3.0 image analysis software (Kinetic Imaging Ltd., Liverpool, UK).

Tail moment (tail-extent moment; *TM*) was defined as: $TM = I \times L$ , where

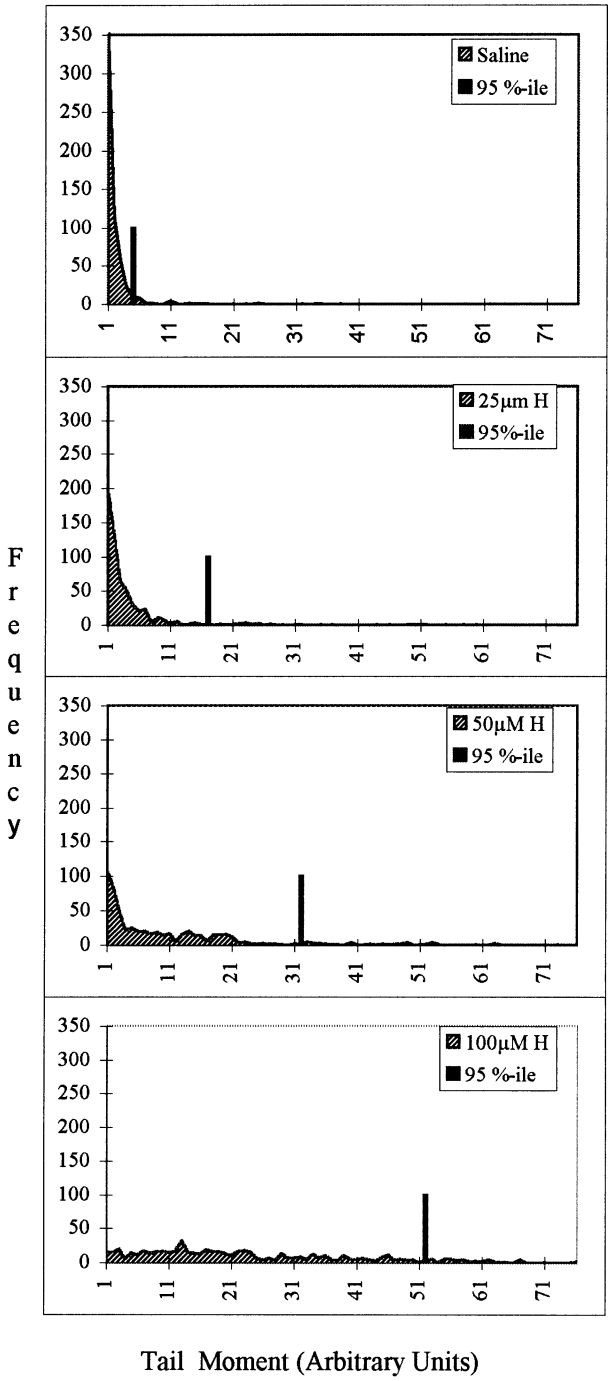


**Fig. 1.** Frequency distribution of tail moments from 600 CACO-2 cells treated with hydrogen peroxide using the comet assay: 95th percentile (95%-ile) cut-off values determined for genotoxicity screening after treatment with hydrogen peroxide (H) at 0 (saline), 25, 50 and 100 µM concentrations.

*I* is the fractional amount of DNA in the comet tail and *L* is the full extent of comet tail from the centre of the comet head to the end of the tail (20).

Endonuclease III (50 µl) was applied to the cells after embedding in agarose on microscope slides. Controls with enzyme reaction buffer alone were included and all slides were incubated at 37°C for 45 min.

*Statistical methods for comet data analysis*

The 75th percentile (upper quartile) value for tail moment was calculated from 100 cells per slide and provided a single value that gave an appropriate representation of the distribution of the data for statistical analysis. The upper quartile value was used in preference to the mean, since a mean value for this type of data is influenced by the few heavily damaged cells in the

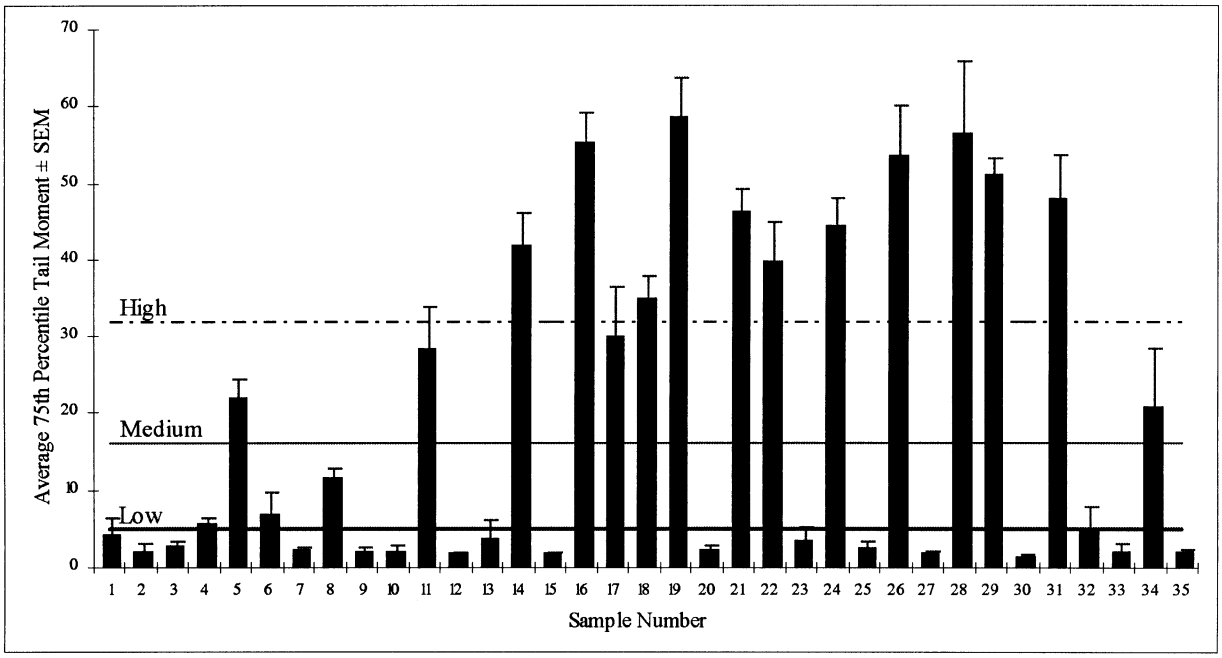


**Fig. 2.** Faecal water screening of a randomly selected group of 35 healthy volunteers. Lines represent 95th percentile cut-off values for: low (▬▬), medium (▬▬), and high (—·—·-) genotoxicity. Sample values are average 75th percentile tail moments ± standard error of the mean (SEM).

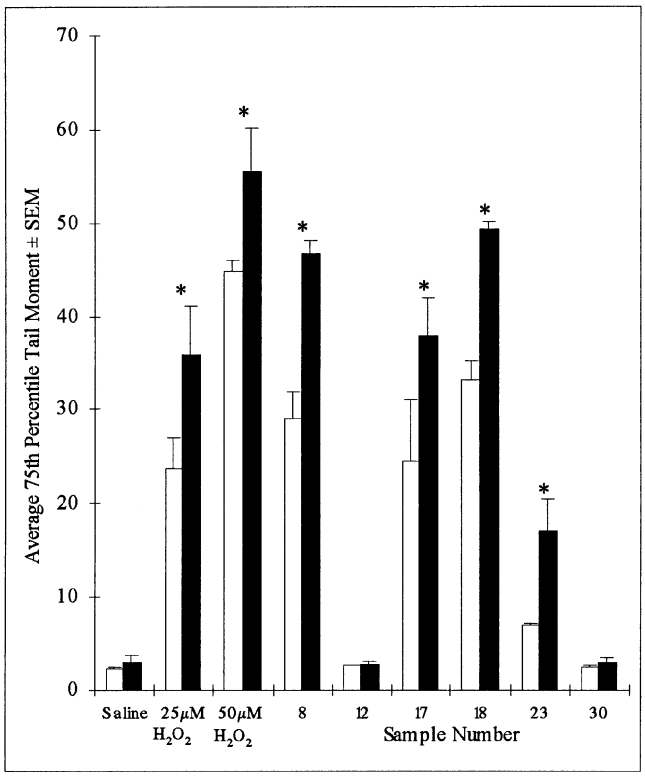


**Fig. 3.** Effect on genotoxic activity in CACO-2 cells of *Escherichia coli* endonuclease III in the comet assay after $H_2O_2$ treatment as a positive control for induction of oxidative damage and using representative faecal waters: □, without endonuclease III; ■, with endonuclease III. Values are average 75th percentile tail moments ± standard error of the mean (SEM). *Significantly elevated tail moments after endonuclease III treatment ($P < 0.05$).

population and does not reflect how the majority of cells respond. For toxicological comparisons, 95th percentile values were determined from six independent experiments; these values were derived to provide upper limits for classification purposes only. The Student's *t*-test was used to compare the genotoxicity of English and Swedish samples, and a paired *t*-test was used to evaluate differences before and after treatment with endonuclease III.

## Results

### *Subject data*

For the Swedish group, 12 volunteers (six men and six women) with a mean age of 40 years (range 25–57 years) participated in the study. For the English group, eight men and 15 women participated; the mean age was 34 (range 17–46 years). There was no apparent correlation between DNA damage assessed as comet tail moment, and age of volunteer, faecal pH, faecal weight or the volume of faecal water per gram faeces. For Swedish and English volunteers, the weights of faecal collections were not significantly different; with mean weights of 117 g (range 67–198g) and 107 g (range 3.7–266 g), respectively. The volume of faecal water per gram faeces and the pH was not significantly different between the Swedish and English groups: The mean volume of faecal water was 200 μl per g faeces, ranging from 18 to 437 μl, and the pH of faeces ranged from 6.1 to 7.5 with a mean value of 6.7.

### *Assessment of genotoxic cut-off points*

For assessing the level of genotoxicity, cut-off points were categorized according to damage induced by $H_2O_2$ as non, low, medium and high genotoxicity (Figure 1). Upper limits were assigned based on 95th percentile values (see Materials and methods). Thus, the 75th percentile tail moment greater than the upper limit for saline (5 units) was selected to indicate low levels of genotoxicity. Hydrogen peroxide (25, 50 and 100 μM) provided a dose-response curve which indicated levels of genotoxicity with lower limit values of 5 units (low), 17 units (medium) and 32 units (high), respectively (Figure 1). It should be noted that this scoring scheme, like other scoring schemes used to categorise comet data, is arbitrary. For a sample to be allocated to a genotoxic category, the average 75th percentile tail moment value must be in the appropriate region (i.e. between 5 and 17 units for low

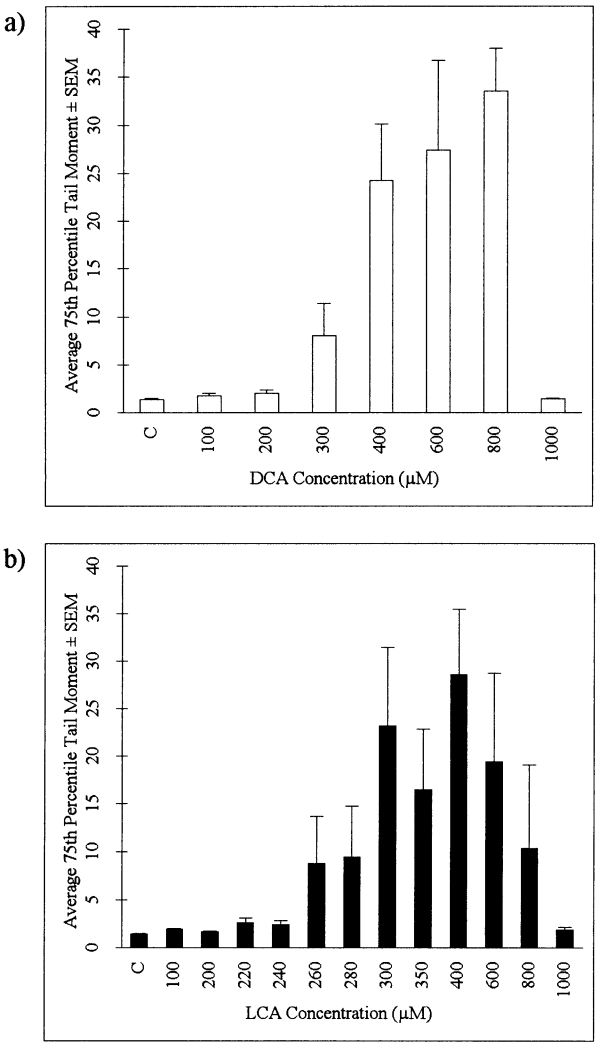


**Fig. 4.** Genotoxicity of bile acids to CACO-2 cells as assessed by the comet assay: (**a**) DCA (□); (**b**) LCA (■). Values are average 75th percentile tail moments ± standard error of the mean (SEM).

genotoxicity), and samples were placed in the lower group if the value was close to the cut-off limit and the SEM extend below the cut-off limit.

*Viability assessment*

Viability was assessed by trypan blue exclusion for all assays. Viability of CACO-2 cells was consistently >90%, and the viability of treated cells was not significantly different to control cells (data not shown).

*Assessment of the optimum concentration of faecal water*

There was a dose-dependent increase in tail moments when the faecal water concentration was increased from 4 to 10% (data not shown), with a higher number of genotoxic samples detected at the higher concentration. The 10% dilution of faecal water was chosen for use in the main study.

*Faecal water screening*

Figure 2 summarizes results of the faecal water screening from the randomly selected group of 35 healthy volunteers. Swedish samples were numbered 1–12 and English samples numbered 13–35. Low genotoxicity levels were selected above tail moments of 5 units based on negative control (saline) data, and categorized as non, low, medium and highly genotoxic

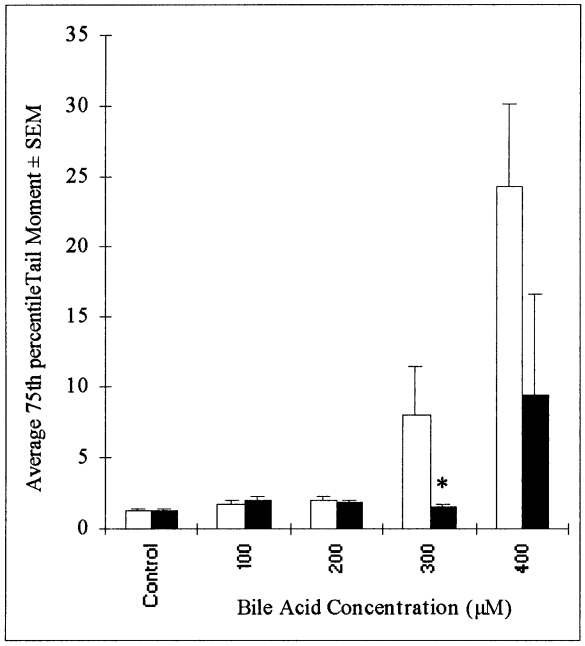


**Fig. 5.** Differences in genotoxic activity in the comet assay between DCA free acid and its sodium salt: □, DCA-free acid; ■, DCA sodium salt. Values are average 75th percentile tail moments ± standard error of the mean (SEM). *Significantly lower tail moment ($P < 0.05$).

as outlined above. Sample 8 was the only sample of low genotoxicity; samples 5, 11, 17 and 34 were the four samples in the medium category, and samples 14, 16, 18, 19, 21, 22, 24, 26 28, 29 and 31 were the 11 highly genotoxic samples. The remaining 19 samples were non-genotoxic. Overall, 43% of faecal waters were in one of the genotoxic categories.

There were some differences in genotoxicity between Swedish and English faecal samples; the mean tail moment values for Swedish samples (7.68 ± 2.52; mean ± SEM) was significantly lower than for the English samples (26.47 ± 4.77; $P < 0.05$): 56% of the English samples screened (13 of 23) were genotoxic, whilst only 25% of the Swedish samples screened (four of 12) were genotoxic. Those English faecal waters that were genotoxic also showed a higher level of activity than the Swedish samples (Figure 2).

The water content of the faeces from volunteers varied widely and there was no correlation between faecal water volumes and genotoxicity in our assay (data not shown).

*Effects of endonuclease III*

The effects on genotoxic activity of *Escherichia coli* endonuclease III were investigated using $H_2O_2$ treatment on CACO-2 cells as a positive control for induction of oxidative damage. There was no significant increase in DNA damage in saline controls after incubation with the enzyme; however, significantly increased tail moments ($P < 0.05$) were obtained after endonuclease III treatment with 25 and 50 μM $H_2O_2$ (Figure 3).

A range of genotoxic and non-genotoxic faecal water samples from the screen were selected to investigate the effects of endonuclease III (Figure 3). There was no significant increase in DNA damage for the two samples which were non-genotoxic in the comet assay (samples 12 and 30). There was a significant increase ($P < 0.05$) in the tail moments from genotoxic samples treated with endonuclease III: the enzyme increased the 75th percentile tail moments for low-, medium- and highly-genotoxic samples (23, 17 and 18, respectively).

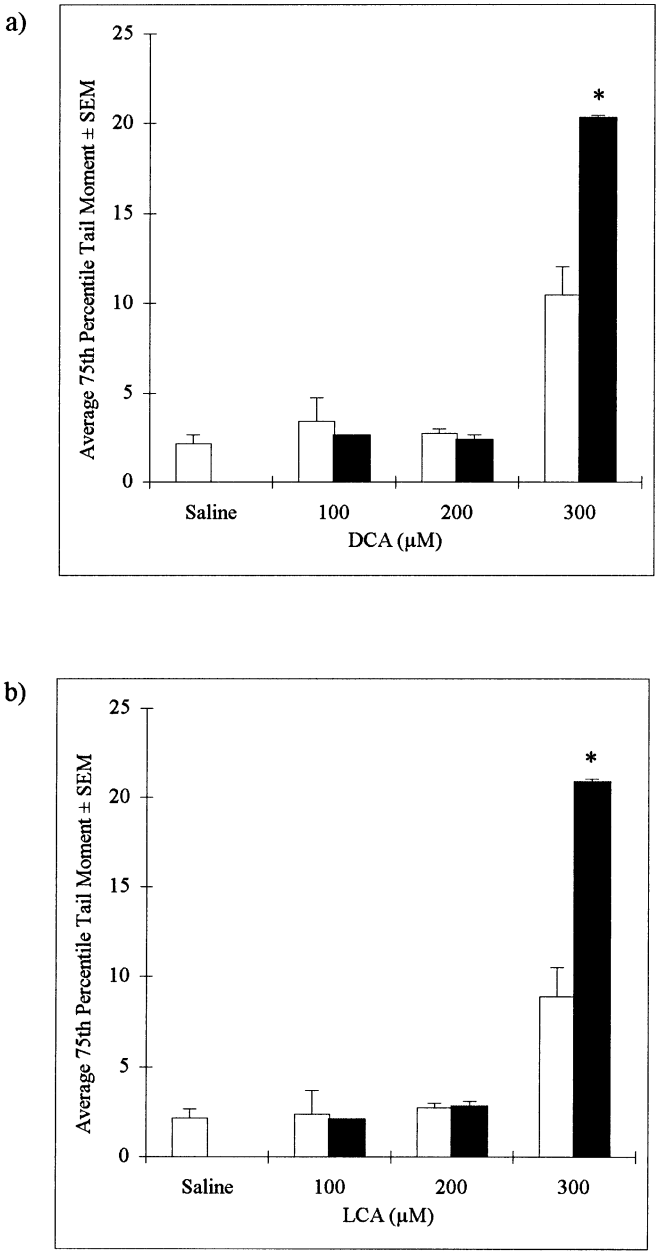


**Fig. 6.** Effect of endonuclease III on genotoxicity of bile acids to CACO-2 cells in the comet assay: (**a**) DCA; (**b**) LCA. □, Without endonuclease III; ■, with endonuclease III. Values are average 75th percentile tail moments ± standard error of the mean (SEM). *Significantly elevated tail moments after endonuclease III treatment ($P < 0.05$).

*Bile acid genotoxicity*

For LCA there was no genotoxicity up to a concentration of 200 μM, a dose-dependent increase in genotoxicity from 260 to 400 μM, after which LCA precipitated from solution and its DNA-damaging effects were lost (Figure 4). For DCA up to 200 μM there was no increase in tail moment, but a dose-dependent elevation of tail moments occurred from 300 to 800 μM, above which DCA precipitated from solution and the DNA damaging effects detectable in the comet assay were lost (Figure 4).

No significant increase in tail moments were observed for the free acid or sodium salt of DCA up to 200 μM; however, tail moments were lower ($P < 0.05$) for the sodium salt compared with the free acid at 300 μM (Figure 5).

*Effect of endonuclease III on bile acid genotoxicity*

After endonuclease III treatment there was no elevation of the tail moments for DCA or LCA up to 200 μM; however, at 300 μM both DCA and LCA tail moments were significantly higher ($P < 0.05$) in the presence of the enzyme (Figure 6).

## Discussion

The results of the present study indicate that the comet assay provides a powerful tool to investigate the genotoxicity of faecal water samples in a human colonic tumour cell line. Some limited studies in mucosal cells isolated from the rat colon indicate that genotoxic activity could be detected in primary cultures as well as cell lines (I.R.Rowland and A.Smedman, unpublished observations). Cell lines were considered preferable as they obviated the need to use animals and provided a human target cell. Cell lines from normal human colon are not available and the use of the CACO-2 human colonic adenocarcinoma cell line in this system would appear to provide a relevant, currently available model for genotoxic assessment in the human colon. CACO-2 cells undergo spontaneous enterocytic differentiation in confluent monolayer cultures, and exhibit many characteristics of normal epithelial cells including the excretion of brush border-associated enzymes (21), well defined apical brush borders, and tight intercellular junctions (22). The model opens up a wide range of possibilities for assessing changes in the genotoxicity of human faeces after dietary intervention.

The results show for the first time that this faecal fraction contains elements able to induce DNA damage. Of the 35 faecal water samples screened using the comet assay, 43% possessed genotoxic activity based on our scoring scheme. The level of induced DNA damage varied considerably between samples; from samples which induced tail moments only slightly above the level of saline controls, to faecal water samples which induced very high tail moments which could be classified as highly genotoxic. It was interesting to note apparent differences between Swedish and English volunteers: Only 25% of the faecal waters from the Swedish group were genotoxic, whilst 56% of the English samples showed significant genotoxicity, and at considerably higher levels (Figure 2). Although the number of individuals screened was low ($n = 35$), these results may suggest that, in healthy volunteers consuming their habitual diet (30% energy from fat for the Swedish samples and 37% for the English samples), there may be differences in faecal water genotoxicity between groups of people from different countries. It is important to note, however, that the samples size from this study was very low (only 12 Swedish samples were obtained) and the differences noted should be investigated in a much larger-scale study before a significant comparison could be made. Should these differences exist, they may be dependent on international variations in dietary intake and gastro-intestinal function and thus warrant further investigation. The assessment of faecal water genotoxicity may provide an important biomarker to investigate associations between diet and cancer.

*Escherichia coli* endonuclease III is an iron-sulphur protein (23) with both AP-endonuclease and N-glycosylase activities, and plays a fundamental role in bacterial DNA-repair systems. EndIII has been purified and characterized (24) and has been shown to be extremely specific for oxidized pyrimidines (15), nicking the DNA at sites of oxidative damage and allowing the detection in the comet assay of DNA damaging agents

which may not simply produce single or double strand breaks as a result of their activity.

Unlike freshly isolated lymphocytes (15), freshly harvested CACO-2 cells (i.e. cells not treated with oxidative reagents like $H_2O_2$) did not show increased DNA damage after treatment with EndIII suggesting that there was no endogenous oxidative damage in this tumour cell line. For faecal waters with no genotoxic activity (samples 12 and 30), there was also no increase in tail moment after EndIII treatment suggesting that these samples did not contain detectable levels of oxidative DNA damaging agents. However, for the low (sample 23), medium (sample 17) and highly genotoxic samples tested (sample 18) there was a significant increase in genotoxicity in the presence of EndIII highlighting at least some level of oxidative damage to the DNA.

Secondary bile acids have been associated with colon cancer and with other common gastro-intestinal diseases (25). A number of studies have shown that the predominant secondary bile acids are cytotoxic to human cell lines (16,26). Using a red blood cell lysis assay, Rafter *et al.* (4) demonstrated that the cellular toxicity of faecal water and bile acid concentration strongly correlates with a high-fat diet. Indeed, diet-induced increases in colonic bile acid concentrations have been shown to lead to lytic activity of faecal water and proliferation of colonic cells (6), and at high physiological levels, bile acids can induce apoptosis in epithelial sub-populations, selecting an 'apoptosis-resistant' population and increasing the likelihood of cancer-prone cells (27).

Whilst much is known about the cytotoxicity of bile acids, there is little genotoxicity data available, with the exception of bacterial mutagenicity tests which have proved contradictory (28,29). The DNA-damaging activity of DCA and LCA in the comet assay follows a dose–response curve which shows an increase in genotoxicity above 300 μM [compared with an $LC_{50}$ cytotoxicity value of 200 μM for DCA (26)]. Thus, the genotoxicity of faecal water samples, observed in the present study, is unlikely to be due to the bile acids, DCA and LCA, as the concentrations of these components would not be expected to reach 300 μM in normal faecal waters (4,30–32). However, of considerable interest in this regard, is a report by Stadler *et al.* (33), where they showed that the concentrations of both DCA and LCA could exceed 300 μM in faecal waters from patients with colonic polyps. Bile acids have previously been shown to induce single-strand breaks in isolated nuclei (34) and the present study has shown the presence of DNA strand breaks and base oxidations at high bile acid concentrations; concentrations that may be reached in patients with colon cancer-associated pathology, but are unlikely to be responsible for the DNA damaging effects seen in the faecal water samples from the present study. Bile acids can also alter colonic cell proliferation (35–37), and the predominant bile acids, in both un-conjugated and conjugated forms can produce DNA adducts *in vitro* (38). Our observation of the much reduced genotoxicity by the sodium salt of DCA when compared to the free acid was surprising, but could be due different rates of uptake through the cell membrane. It has been reported that conjugated bile acids are considerably less cytotoxic to colonic cells than the corresponding free acids (26).

Since bile acid concentrations seem unlikely to account for the genotoxicity of faecal water, other compounds must be responsible for the DNA strand breaks and oxidative damage.

Recently, there have been claims about a possible role of fecapentaenes, especially fecapentaene-12, in mediating genotoxicity by an oxidative mechanism (39,40). In these *in vitro* studies the DNA damaging mechanism appeared to involve oxygen-radicals or direct DNA alkylation. It is not yet known whether fecapentaene excretion differs between populations at varying risk of colorectal cancer. However, one report has shown that 82% of North-Americans excrete detectable levels of fecapentaenes (41). In addition to fecapentaenes, there are a host of other candidate compounds which occur in the faeces that could be responsible for inducing DNA strand breaks. These include nitroso compounds which can be formed in the large bowel from nitrate and nitrite-containing compounds, and the production of oxygen radicals in the faeces.

In conclusion, in view of the increased interest in human faecal water in studies on mechanisms underlying the dietary aetiology of colon cancer, our finding, for the first time, of genotoxic activity in this faecal fraction becomes interesting. Further validation of this model is now required which could lead to the development of a meaningful biomarker for investigating associations between diet and colon cancer.

Thus, exploiting the comet assay work will continue to elucidate the causative agents and mechanisms of DNA damage brought about by this human faecal fraction.

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