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In vivo cytogenetic effects of natural humic acid

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As humic compounds are naturally widespread in the environment and present in surface water, studies on their genotoxicity are justified. Humic acid (HA) has not been demonstrated to be genotoxic either *in vitro* or *in vivo*. In the present paper we investigated its activity both in intestinal and bone marrow cells following a single dose (100 mg/kg b.w. corresponding to 0.5 ml per animal of an aqueous solution of 4 g/l) of HA administered to mice by gastric intubation, to mimic the most likely route of human exposure. HA induced structural and, in particular, numerical chromosome abnormalities in intestinal cells. A marginal, non-significant induction of aneuploidy was also found in bone marrow cells.

Introduction

Humic and fulvic acids, which are by-products of soil organic matter degradation, are naturally present in the environment (soil and water). Their chemical composition is complex and is still under investigation, although resorcinol, vanillic acid, ferullic acid and benzoic acid represent recurrent components (Sato *et al.*, 1987). Humic compounds have been extensively examined (primarily in bacterial systems) since the mutagenicity of chlorinated waters was discovered. Mutagenicity observed in organic material obtained from water extraction (Kool *et al.*, 1981) can be reproduced by chlorinating aqueous solutions of humic compounds (Kowbel *et al.*, 1986; Hemming *et al.*, 1986; Meier *et al.*, 1986; Bernacchi *et al.*, in press). Therefore interest has focused on 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), which derives from the reaction of chlorine with natural organic substances. MX has been shown to be the main factor responsible for humic acid (HA) mutagenicity in *Salmonella* (Hemming *et al.*, 1986; Meier *et al.*, 1987; Kronberg and Vartiainen, 1988), in Chinese hamster ovary (CHO) and in V79 cells (Meier *et al.*, 1987; Brunborg *et al.*, 1991). MX was negative in the micronucleus test in mouse bone marrow (Meier *et al.*, 1987b; Tikkanen and Kronberg, 1990), however, nuclear abnormalities were observed in the gastrointestinal tract of the B6C3F1 mouse after oral administration of MX, suggesting a direct effect at the site of administration (Daniel *et al.*, 1991).

On the other hand, little is known on the genotoxicity of HA itself. Because of its chelating properties HA has mainly been studied for possible antimutagenic activity (Sato *et al.*, 1986, 1987; Cozzi *et al.*, 1993). HA showed only weak mutagenicity and toxicity in human peripheral lymphocytes (Cozzi *et al.*, 1993) and no data are available *in vivo*.

In the present work we analysed the mutagenicity of HA in mouse intestinal cells following *per os* treatment. The advantages of this system are 2-fold: (i) it mimics the most likely route of human exposure to HA, and (ii) since intestinal cells are directly exposed to genotoxins, interspecies variations in pharmacokinetics are reduced (Daniel *et al.*, 1991).

The cytogenetic effects in bone marrow cells were also investigated in order to assess the possibility that active forms of HA could spread through the body and reach this target.

Materials and methods

Preparation of HA solution

In order to solubilize HA (FLUKA), an aqueous solution (4 g/l) was first alkalized to pH 12.0 and subsequently acidified to pH 6.0. The pH was then adjusted to 7.0 and the solution was magnetically stirred for 24 h. Finally the solution was ultracentrifuged twice with a Beckman J2-21 centrifuge for 30 min at 12·800 r.p.m. and sterilized with 0.45 μm pore diameter filters (Flow Laboratories); the pH was adjusted to 7.4 with phosphate buffer.

Animals

Male CD1 Swiss mice, aged 30 days and weighing ~20 g, were obtained from Charles River (Calco, Como, Italy). The animals were housed in disposable polystyrene cages and maintained under conditions of controlled temperature, humidity and lighting, with free access to water and standard laboratory diet prior to use. The animals were allowed 1 week's acclimation period before beginning the experiments.

Animal treatment, slide preparation and scoring

Mice received a single dose of 100 mg/kg b.w. of HA (i.e. 0.5 ml of the 4 g/l aqueous solution), via a stomach tube. For the preparation of intestinal epithelium metaphase cells we followed the procedure described by Neal and Probst (1984) and already adapted for skin epithelium cells (Barale *et al.*, 1992).

Animals were killed by cervical dislocation after 12, 24, 48 and 72 h in order to analyse the temporal sequence describing the temporal appearance of different genetic endpoints. The 72 h recovery was not performed for the positive control. The ileum and duodenum were removed from the animals, placed in small Petri dishes and carefully rinsed in cold physiological NaCl solution (0.9%). Then they were cut in very small fragments and placed in hypotonic solution (KCl, 0.075 M) for 30 min at room temperature. During this time the hypotonic solution was changed every 10 min. The solution was then removed, pieces were dried with blotting paper and then fixed with cold methanol:acetic acid (3:1) for 1 h at −20°C. After fixative removal, tissue fragments were dissociated in a glacial acetic acid (GAA) solution (60%). After 10–15 min cells start being released from the edge of the tissue pieces. Sample suspensions containing interphase and metaphase cells were dropped onto warmed (80°C) slides every 30 min up to 2–3 h. During this time, intestine pieces continue to be softened and disaggregated by GAA, releasing inner metaphase cells. After each sampling, fresh GAA was added to maintain constant the incubation volume in the Petri dish. Slides were immediately observed under a phase contrast microscope in order to assess the presence of a suitable number of scorable metaphases. Dried slides were Giemsa-stained (5% for 10 min) and mounted in Eukitt.

Bone marrow cells were removed from the femur and processed according to standard procedures for metaphase chromosome scoring (Adler, 1984).

Negative controls which received an oral administration of the vehicle used (water) were performed at each recovery time. Pure water was chosen as control because, in our experimental conditions, alkalization (NaOH) and acidification (HCl) of HA solution produced a very small quantity of NaCl, which was thought to be unlikely to affect the results. Methyl-2-benzimidazole carbamate (MBC; Du Pont De Nemours, USA, analytical grade), was used as positive control; animals received peroral (p.o.), by gavage, 50 mg/kg b.w. of MBC dissolved in dimethyl sulphoxide (DMSO) and then diluted in water to a final 2% DMSO concentration. MBC solutions were given to each animal

in the volume of 0.5 ml. For each experimental point, three or four animals were used and no fewer than three were analysed; at least 50 well-spread metaphases were scored for each animal for the presence of chromosomal aberrations. Since no differences were observed between ileum and duodenum, obtained data were pooled. Fisher's Exact Test was adopted to assess the statistical significance of treated groups in comparison with controls.

Results and discussion

It has been shown that mouse intestinal cells may be more responsive than bone marrow cells when animals are treated by gastric intubation with direct acting mutagens (Neal and Probst, 1984). However, probably due to technical difficulties, intestinal cells have not been further investigated to study induction of chromosome effects *in vivo*. Actually, it is important to note that intestinal cells represent the first and possibly the most responsive target cells for exposure to water and food genotoxins, while bone marrow may be evaluated as a secondary target tissue for compounds that are absorbed, metabolized, and systemically distributed (Neal and Probst, 1984). Because of interest in the possible genotoxic effect of HA *in vivo*, the methodology proposed by Neal and Probst (1984) was applied to intestinal cells using MBC as a positive control. MBC, the presumed active form of the fungicide benomyl, showed both clastogenic and aneugenic activity in mouse bone marrow at the dose of 500 mg/kg b.w. (Styles and Garner, 1974; Barale *et al.*, 1993). As shown in Table I, MBC was fairly active in the intestine, inducing both structural and numerical chromosome aberrations. Aberrant cells appeared as early as the first sampling time (12 h) and induction of numerical aberrations seemed to persist up to 48 h. These findings, combined with cell kinetic studies (Neal and Probst, 1984), suggest that intestinal cells are comparable with bone marrow cells except for the early appearance of effects due to immediate contact with high doses of the compound administered. All effects were obtained by using a dose (50 mg/kg b.w.) which had no apparent effects far from the site of contact, i.e. in bone marrow cells (Table II). To obtain positive results at this site, 10-fold higher doses are required (500 mg/kg b.w.; Barale *et al.*, 1993). Therefore intestinal cells confirm their reliability when the genotoxicity of direct acting chemicals taken *per os* is under assessment.

The cytogenetic effects of HA in intestinal epithelium and bone marrow cells are shown in Tables III and IV respectively.

HA induced both structural and numerical aberrations in duodenal and ileal epithelial cells. Chromosome effects produced by HA have never been documented, but we found analogous effects on cultured human lymphocytes treated *in vitro* (manuscript in preparation). Induction of aberrant cells was time-dependent, reaching a maximum after 24 h and persisting up to 72 h after animal exposure.

HA appeared to give no statistically significant effects in bone marrow cells. It should be pointed out, however, that an increase in aneuploid cells was observed. Thus if all data are pooled (treated compared with controls) disregarding sampling time, then the increase in aneuploid cells is shown to be statistically highly significant.

The great majority of aneuploid cells were classified as 2n + 1 and, rarely, 2n + 2. This suggested a mild effect on the mitotic apparatus, which was never blocked completely, as

Table I. Structural and numerical chromosome aberrations on intestinal epithelial cells after oral administration of 50 mg/kg of methyl-2-benzimidazole carbamate (MBC)

Recovery time (h)	Metaphases scored	G	B	F	AC (– G)	AC (+ G)	An C
Control 0	400	3	0	1	1	4	0
12	400	8	3	13	16^{***}	24^{***}	11^{***}
24	400	3	1	8	9*	12*	20^{***}
48	400	0	0	2	2	2	16^{***}

G = gaps; B = breaks; F = fragments; AC (– G) = total aberrant cells excluding gaps, AC (+ G) = total aberrant cells including gaps; An C = total aneuploid cells
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Table II. Structural and numerical chromosome aberrations on bone marrow cells after oral administration of 50 mg/kg of methyl-2-benzimidazole carbamate (MBC)

Recovery time (h)	Metaphases scored	G	B	F	AC (– G)	AC (+ G)	An C
control 0	200	1	0	0	0	1	0
12	200	1	0	4	4	5	0
24	200	0	0	2	2	2	1
48	200	1	0	1	1	2	1

G = gaps, B = breaks; F = fragments; AC (– G) = total aberrant cells excluding gaps, AC (+ G) = total aberrant cells including gaps, An C = total aneuploid cells.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table III. Structural and numerical chromosome aberrations on intestinal epithelial cells after oral administration of 100 mg/kg of an aqueous solution of humic acid (HA)

Recovery time (h)	Metaphases scored	G	B	F	AC (– G)	AC (+ G)	An C
12 (control)	150	1	1	0	1	2	1
12 (treated)	150	2	2	1	3	4	5
24 (control)	150	2	1	1	2	4	0
24 (treated)	150	5	7	3	10*	12*	15^{***}
48 (control)	150	2	0	0	0	2	1
48 (treated)	150	4	2	1	3	7	8*
72 (control)	150	2	0	0	0	2	1
72 (treated)	150	1	1	0	1	2	7*

G = gaps; B = breaks; F = fragments, AC (– G) = total aberrant cells excluding gaps; AC (+ G) = total aberrant cells including gaps, An C = total aneuploid cells.
$^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$

Table IV. Structural and numerical chromosome aberrations on bone marrow cells after oral administration of 100 mg/kg of an aqueous solution of humic acid (HA)

Recovery time (h)	Metaphases scored	G	B	F	AC (– G)	AC (+ G)	An C
12 (control)	150	0	0	0	0	0	0
12 (treated)	150	1	0	0	0	1	3
24 (control)	150	2	0	0	0	2	1
24 (treated)	150	1	1	0	1	2	5
48 (control)	150	2	0	1	1	2	0
48 (treated)	150	2	1	0	1	3	4
72 (control)	150	1	1	0	1	1	1
72 (treated)	150	1	0	1	1	1	6

G = gaps; B = breaks, F = fragments, AC (– G) = total aberrant cells excluding gaps; AC (+ G) = total aberrant cells including gaps; An C = total aneuploid cells.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

shown by the fact that polyploid cells were never observed. Hypodiploid cells, although observed, were not considered because they may derive from experimental artefacts (Liang and Satya-Prakash, 1985).

Lignins, rich in -OCH_3 groups, have been identified as one of the main components of HA (Flaig, 1966; Kononova, 1966). These groups, which interfere with tubulin polymerization, are responsible for the aneuploidogenic activity of colchicine, vincristine and vinblastine. A similar action is therefore suggested for HA. In addition, the chelating properties of HA enable it to capture metallic ions useful for the normal process of mitotic division (Onfelt, 1986).

HA showed a clastogenic activity in intestinal cells after 24 h exposure. Such a phenomenon is not well understood; a speculative explanation could be that HA was chlorinated in the gastrointestinal tract, resulting in the formation of MX which has been shown to be clastogenic both *in vivo* (Meier *et al.*, 1987b; Daniel *et al.*, 1991) and *in vitro* (Nunn and Chipman, 1994). Similarly to MBC, HA was not found to be clastogenic in bone marrow cells. Such an effect can be explained by MX distribution, in as much as, outside of blood, MX appears to be restricted to tissues involved in absorption or excretion (Ringhand *et al.*, 1989; Komulainen *et al.*, 1992). Moreover, since MX is relatively unstable in water at neutral pH, it would be expected to undergo rapid detoxification through chemical reactions with macromolecules of blood serum. And similarly to MBC, such limited stability and/or solubility could lead to organ-specific effects (Seiler, 1976; Brunborg *et al.*, 1991). Therefore, MX may not reach bone marrow at concentrations sufficient to cause chromosomal damage (Pakkanen and Jansson, 1995).

These data strongly suggest that HA could exert mutagenic activity at the site of absorption. The different sensitivity observed in bone marrow and small intestine underlines the need to use an intestinal cytogenetic test to investigate the possible effects directly induced by chemicals at the site of administration. However, to obtain sufficiently well spread metaphases from intestinal epithelium as compared with bone marrow involves laborious procedures and requires highly skilled technicians. Consequently this methodology is particularly suitable for implementation of mutagenicity data in cases considered relevant for health risk evaluation.

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