Environmental Health Perspectives
Vol. 69, pp. 141-149, 1986

Mutagenic Activity of Concentrated Drinking Water Samples

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Concentrated drinking water extracts prepared by adsorption onto XAD-2 resin have been tested for their ability to induce chromosome damage in mammalian cells. Extracts prepared from drinking waters derived from upland and lowland sources have been found to induce chromosome aberrations in Chinese hamster ovary (CHO) cells and human lymphocytes *in vitro*. Although the identity of the compounds responsible for this activity is unknown, they are generated when the water is chlorinated and appear to bind readily to exogenous protein.

When groups of mice were dosed orally with a concentrated water extract, however, no evidence of clastogenic activity in bone marrow cells was apparent. The absence of an *in vivo* effect may indicate that the mutagenic compounds failed to reach the bone marrow. The possibility that genetic damage could be induced in the cells first encountered in the body after ingestion (i.e., cells in the esophagus, stomach, and intestinal tract) is not precluded by this result. The relevance of these findings in evaluating the potential health hazard of mutagenic compounds in drinking water is discussed.

Introduction

Many laboratories around the world have reported that concentrated extracts of chlorinated drinking water derived from surface sources show mutagenic activity in bacterial test systems (*1–8*). The compounds responsible for this activity have not been identified, but they appear to be generated when the water is chlorinated for disinfection purposes. The precursor compounds that are transformed to mutagens during chlorination are probably naturally occurring organics in surface waters, such as humic acids and amino acids (*9–11*).

When pure substances are tested for genotoxic activity, it is generally agreed that the compounds should be screened in a battery of test systems, including bacterial, cultured mammalian cell, and whole-animal assays. At the Water Research Centre, we have adopted this approach for evaluating the mutagenic activity of concentrated water extracts and are conducting a range of experiments in higher cell systems. In this paper we report some of the results we have obtained in *in vitro* and *in vivo* cytogenetic assays.

Materials and Methods

Sample Preparation

Samples of final drinking water were collected in stainless-steel containers from water treatment works before the water passed into distribution. In the laboratory, the water samples were pulled through XAD-2 resin columns (20 mL bed volume) at a flow rate of 100 mL/min. The columns were then blown dry with nitrogen and eluted with 50 mL of high-pressure liquid chromatography (HPLC)-grade acetone. The volume of the acetone eluate was reduced to about 7 mL by rotary evaporation and then by gentle evaporation under a stream of nitrogen. For tests with bacteria, these extracts are usually left in a small volume of acetone or exchanged into dimethyl sulfoxide (DMSO). Mammalian cells, however, will tolerate only low concentrations of most organic solvents (2% volume/volume as a maximum) and, consequently, the concentration of the extracts that can be tested may be limited by solvent toxicity rather than by the toxicity of the dissolved organics. In an attempt to prevent this problem, the acetone extracts were evaporated to dryness and reconstituted in deionized/carbon-filtered water such that 1 mL of extract was equivalent to 10 L of original water. These samples were filter-sterilized through 0.45 μm membrane filters and stored at -20°C until testing was completed. Extracts prepared in this way showed clear mutagenic activity in bacterial fluctuation assays, although slightly higher doses of these extracts (in liters of original water/milliliter of test medium) were required than those of the original acetone/DMSO extracts.

Raw water samples were collected either directly from a lowland river or from the intake channel of a water treatment works. At the laboratory, the water was filtered through Whatman GF/C grade filters and concentrated as described previously. For some exper-

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iments, raw water samples were chlorinated in the laboratory with a single dose of 6 mg/L chlorine for a contact period of 2 hr and then dechlorinated with sodium sulfite to leave a free residual of 0.5 mg/L chlorine.

Procedural blanks were prepared from deionized/activated-carbon-filtered water that was chlorinated in the laboratory and concentrated as described above. Blanks were included in each experiment at a single dose level equivalent to the highest concentration of test sample used.

Cytogenetics Assay in Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells were cultured in McCoy's 5a medium containing 15% fetal calf serum (FCS) and 25 mM Hepes buffer and were passaged using 0.05% trypsin/0.02% ethylenediaminotetraacetic acid (EDTA) solution. For the cytogenetics assay, cells were seeded into 25 cm^2-tissue culture flasks at about 2–3 × 10^5 cells/flask and incubated at 37°C overnight. The medium was removed with a vacuum pump and the cells washed with Hanks' balanced salt solution (HBSS). Double-strength McCoy's medium minus FCS (2 mL) was added to each flask and the total volume made up to 4 mL with the appropriate amounts of test sample and double-distilled water. The cells were incubated in the presence of the test material for 3 hr, and then the treatment medium was replaced with normal McCoy's medium + 15% FCS + penicillin/streptomycin. The cells were incubated for a further 21 hr with 2 × 10^{-7} M colcemid present for the last 2 hr to arrest cells in metaphase. Mitotic cells were dislodged by shaking the flasks gently and then collected by centrifuging. The cells were treated with hypotonic solution (0.075 M potassium chloride for 10 min at 37°C and then fixed by three changes in 3:1 methanol/glacial acetic acid. The fixed, cell suspension was spotted onto glass slides, air dried, and stained with 2% Giemsa. The slides were then coded and scored blind for induced cytogenetic damage (100 metaphases per treatment).

It has been shown previously, using bacterial assays, that the mutagens produced during the chlorination of surface waters are direct-acting, and that if rat liver S9 fraction is included during the treatment period, activity is reduced. For the cytogenetic experiments using CHO cells, treatments were therefore carried out only in the absence of S9.

Human Lymphocyte Assay

For the human lymphocyte assay, blood cultures were prepared using fresh, heparinized blood. These cultures contained 9.5 mL Ham's F10 medium + 15% FCS, 0.5 mL of whole blood, and 50 μL of phytohemagglutinin (1 mg/mL). These cultures were incubated at 37°C for 48 hr. The cells were then centrifuged, washed in phosphate-buffered saline (PBS), and resuspended in 2.5 mL of double-strength serum-free medium. The total volume was made up to 5 mL with the appropriate amount of test sample and doubly distilled water, and the cultures were then incubated at 37°C for 3 hr. After this time, the cells were returned to normal medium + 15% FCS + penicillin/streptomycin, and incubation was continued for a further 21 hr with colcemid present for the last 3 hr. Slides were prepared as described for the CHO assay.

In the first lymphocyte assay, the concentrated extract was tested in the presence and absence of Aroclor-induced rat liver S9 fraction. Ten percent S9 mix (containing 15% v/v S9 fraction) was included in the treatment medium during the 3-hr exposure to the test sample.

For the experiments with separated lymphocytes, whole blood was diluted 1:1 with PBS and layered into 10 mL centrifuge tubes containing 6 mL of lymphocyte separation medium (Flow Laboratories). The tubes were centrifuged for 30 min at 400*g*, and then the lymphocytes were collected from the interface of the media. The cells were washed three times in PBS and then used to inoculate 10 mL cultures as described for whole blood. The cells were grown for 72 hr before treating for 4 hr in serum-free medium. All other aspects of the method were as described for whole-blood cultures.

In Vivo Study

To investigate the clastogenic activity of a concentrated water extract *in vivo*, groups of mice were fasted for 10 hr and then orally administered concentrated drinking water extract derived from a lowland river. The mutagenic activity of the extract in both bacteria and CHO cells *in vitro* was confirmed before the *in vivo* study was begun. The doses used were equivalent in milligrams/kilogram to the total drinking water intake of a 60 kg man drinking 2 L per day for 10 years, 3 years, or 1 year. On this basis, for the highest dose group (i.e., the equivalent exposure for 10 years), a 30-g mouse received a single dose of concentrated extract equivalent to approximately 3.1 L of water. The experiment also included a negative control group, a procedural blank (administered at the same volume as the highest treatment group) and a positive control (a single oral dose of 5 or 10 mg/kg mitomycin C). Three groups of 10 mice (5 males and 5 females) were used at each dose level, and one group was sacrificed at 6, 24, and 48 hr after treatment. At 1.5 hr before sacrifice, the animals were given an intraperitoneal injection of 0.01 mL/g body weight of colchicine (0.04% solution in distilled water) to arrest cells in metaphase.

The two femurs (thigh bones) were dissected out separately and the bone marrow flushed out with HBSS. The cells were treated with hypotonic solution, fixed in methanol/acetic acid, spotted onto microscope slides, and stained with Giemsa. The slides were coded, and 50 metaphases were scored from each femur (i.e., 100 cells per animal) for the 24-hr sampling time, and 25 metaphases were scored per femur for the animals sacrificed after 6 or 48 hr.

Table 1. Percentage of CHO cells with aberrations treated with a laboratory-chlorinated lowland river sample (X019).

Treatment	Number of metaphases analyzed	Cells with aberrations, %[a]		
		Chromatid	Chromosome	Total
X019, 1 L/mL	100	75 (72)	31 (30)	79 (76)
X019, 0.5 L/mL	100	13 (7)	8 (8)	20 (14)
X019, 0.25 L/mL	100	9 (1)	1 (1)	9 (2)
Procedural blank 1 L/mL	100	2 (0)	4 (3)	6 (3)
MNNG, 1 μg/mL	100	88 (84)	27 (27)	91 (89)
Untreated control	100	10 (1)	3 (3)	13 (4)

[a] Figures in parentheses exclude cells with gaps only.

Analysis of Results

In view of the potent clastogenic activity of concentrated water extracts in CHO cells, we considered a statistical analysis of these data unnecessary. For the human lymphocyte assays and the *in vivo* mouse bone marrow study, the results were analyzed for statistical significance using Fisher's exact probability test.

Results

Cytogenetic Assays in CHO Cells

In the first CHO experiment, a concentrated extract prepared from a lowland river sample chlorinated in the laboratory was tested for clastogenic activity in CHO cells. The doses used in this study were equivalent to 0.25, 0.5, and 1.0 L of original water/mL of test medium. A full procedural blank was also included in the experiment, along with appropriate positive and negative control groups.

The results of this experiment are given in Tables 1 and 2. Table 1 gives the percentage of cells containing chromatid or chromosomal-type aberrations, and Table 2 shows the total number and type of aberrations observed with each treatment. At a dose level of 1 L/mL, sample X019 showed potent clastogenic activity in CHO cells, with 79% of the cells containing some form of chromosome damage. An increase in both chromatid and chromosomal-type aberrations was observed. These aberrations included gaps, breaks, fragments, and interchanges. At a dose of 0.5 L/mL, weak clastogenic activity was observed, whereas at 0.25 L/mL, the frequency of aberrations was comparable to that of the untreated control group. No evidence of clastogenic activity was found for the procedural blank, indicating that the mutagenicity of X019 was not an artifact generated during the concentration procedure. Cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), the positive control, showed a high level of chromosome damage with 91% of the cells examined containing some form of aberration.

In view of these results, a repeat experiment was conducted using another laboratory-chlorinated sample (X025) prepared from the same raw water source. In this experiment, dose levels equivalent to 0.5, 0.75 and 1.0 L of original water/mL of test medium were used. The results of this assay (data not shown) confirmed the clastogenic activity of these water extracts. In fact, with this sample, slightly higher levels of chromosome damage were observed than in the previous study. In this experiment, the procedural blank was tested up to a dose of 3 L/mL and still showed no evidence of clastogenic activity.

The next series of experiments were performed on extracts prepared from chlorinated final drinking waters collected from water treatment works. Two samples (X021 and X063) were derived from a lowland river that received both industrial and sewage effluent; the third water sample (X035) was a final water derived from a reservoir that was situated in an upland area of the country, away from any major source of pollution. The results given in Table 3 show the percentage of cells containing chromosome aberrations after treatment with these extracts. All three samples were clastogenic in CHO cells, producing a wide range of different types of aberrations, including a large number of interchanges, which are generally regarded as the most significant form of damage (*12*). The final water extracts were slightly less active than laboratory-chlorinated samples in terms of the dose required to give a positive effect, and the lowland samples appeared to be more potent than the upland sample. The dose responses observed with the two lowland samples (which were taken from the same sampling point but 14 months apart) were remarkably similar, with 55% of the cells showing damage at a dose of 2 L/mL for both samples. Photographs of damaged cells following treatment with these extracts are shown in Figures 1 and 2.

To investigate the origin of the clastogenic agents, concentrated extracts were prepared from raw water taken from the intake of a water treatment works and from the final water leaving the works. Two treatment works were studied, one of which used a lowland river as a water source (samples X036), and the other, an upland reservoir (samples X071). A summary of the results obtained in these experiments is given in Table 4.

The raw water from the lowland river showed evidence of weak clastogenic activity at doses above 1.5 L/mL. The types of aberrations induced were primarily chromatid breaks. In addition to inducing a low frequency of chromosome aberrations, the sample appeared to have an effect on chromosome condensation. In treated cells, the chromosomes were short and "fuzzy" in appearance, and the chromatids were often separated (see Fig. 3). This effect appeared to be dose related, and at the higher concentrations examined, it was difficult to find metaphases that were suitable for scoring. This is the type of response one might expect if cells were exposed for a prolonged period to a compound that disrupted the mitotic spindle, causing the cells to be arrested in the metaphase stage of cell division.

The chlorinated water sample showed much more potent clastogenic activity than the raw water sample, inducing a complete range of chromatid and chromo-

Table 2. Types of aberrations observed in CHO cells treated with a laboratory-chlorinated lowland river sample (X019).[a]

Treatment	Number of cells with multiple damage[b]	Number of aberations in 100 cells[c]									
		Chromatid					Chromosomal				
		G	B	I	Fr	Id	G	B	D	AF	R
X019 1 L/mL	18	27	39	80	11	14	1	14	4	18	5
X019 0.5 L/mL	0	6	1	6	1	0	0	6	1	1	0
X019 0.25 L/mL	0	9	1	0	0	0	0	1	0	0	0
Procedural blank 1 L/mL	0	2	0	0	0	0	1	3	0	0	0
MNNG 1.0 μg/mL	27	8	18	94	12	10	0	9	6	18	7
Untreated control	0	9	1	0	0	0	0	1	0	3	0

[a] G, gaps; B, breaks, I, interchanges; Fr, fragments (single); Id, interstitial deletions; D, dicentrics, AF, acentric fragments; R, rings.
[b] >10 aberrations per cell.
[c] Excluding aberrations in cells with multiple damage.

Table 3. Percentage of CHO cells with aberrations following treatment with final drinking water extracts derived from a lowland river (X021 and X063) and an upland reservoir (X035).

Dose of extract, L/mL	Cells with aberrations, %[a]		
	X021	X063	X035
4.0	—	—	85 (78)
3.5	—	—	72 (69)
3.0	—	—	53 (50)
2.5	—	—	41 (38)
2.0	55 (48)	55 (47)	28 (21)
1.75	46 (33)	53 (44)	—
1.5	22 (14)	30 (24)	—
1.0	18 (13)	23 (12)	11 (2)
0.5	14 (8)	21 (10)	—
Control	9 (6)	20 (16)	6 (4)
Procedural blank	8 (7)	15 (6)	13 (5)
MNNG (1 μg/mL)	77 (74)	—	72.5 (72.5)

[a] Figures in parentheses exclude cells with gaps only.

somal-type aberrations. At a dose of 2 L/mL, 94% of the cells contained some form of damage. An effect on chromosome condensation was also noted with the chlorinated sample, but this was less apparent than with the raw water sample. Since this effect was not observed with the samples from an upland reservoir, it is possible that this condensation effect was not related to the mutagenic activity but to the presence of a specific pollutant in the raw river water. If this was the case, it would appear that passage through the treatment works was at least partially effective at removing the substance(s) responsible for this effect.

The raw water from the upland reservoir also produced a slight increase in the frequency of chromosome aberrations, including a few interchanges that are seldom observed in control cultures. The origin of the compounds responsible for this weak activity is unknown but could include runoff from surrounding agricultural land or naturally occurring mutagens, such as bracken toxins (*13*). The final chlorinated water showed much more potent activity, with 89% of the cells showing damage at a dose of 3 L/mL. These results, therefore, would appear to confirm the findings of previous mutagenicity studies, which show that the major portion of mutagenic activity in drinking water samples is produced when the water is chlorinated.

In our preliminary investigations on the clastogenic activity of water extracts, we noted that the presence of serum in the medium during treatment appeared to reduce the activity of the samples. To verify this observation, we conducted an experiment in which a concentrated water sample was tested at a single dose (1.75 L/mL) in the presence of 0, 2, 5, 10, and 15% FCS in the treatment medium. The water extract (X036) was prepared from a final drinking water derived from a lowland river. A previous experiment had shown that a concentration of 1.75 L/mL induced a high frequency

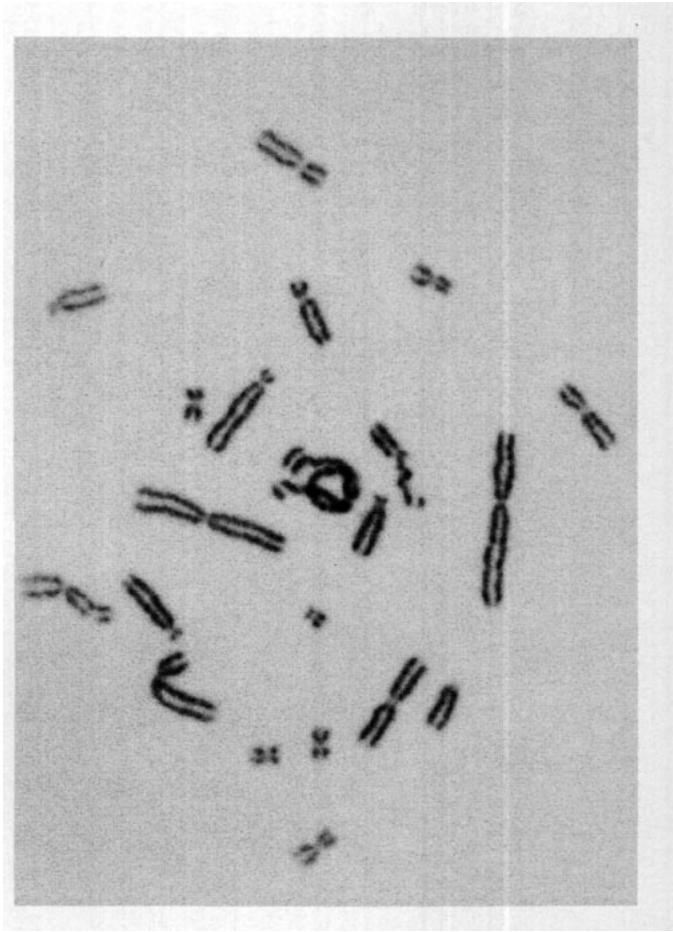

FIGURE 1. CHO cell treated with a concentrated drinking water extract (2 L/mL) showing a chromatid intrachange and chromatid breaks.

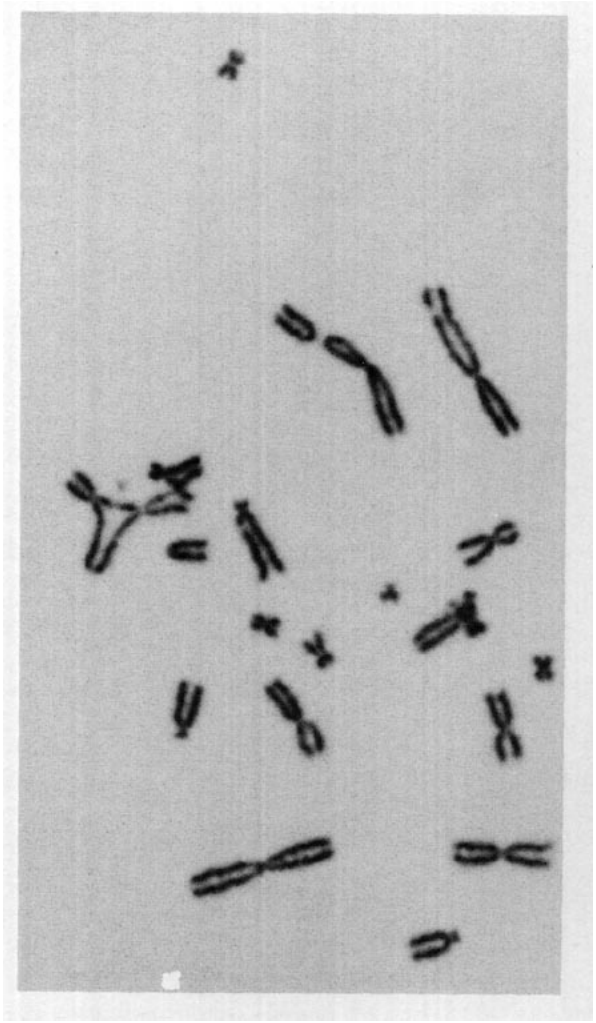

FIGURE 2. CHO cell treated with a concentrated drinking water extract (2 L/mL) showing a chromatid interchange, chromatid gaps, and a chromosome break with sister union of broken ends.

of aberrations. The results of this experiment are summarized in Table 5. The level of chromosome damage observed was inversely proportional to the concentration of serum in the treated medium. With no serum, 86% of metaphases showed some form of aberration, whereas in the presence of 15% serum, this figure was reduced to 24%. However, even with the high serum levels, the frequency of aberrations was still significantly higher in the treatment cultures than in the corresponding controls. The presence of serum, therefore, reduced, but did not abolish, the clastogenic activity of the sample. It is interesting to note that the presence of serum also reduced the clastogenic activity of the positive control (MNNG), but the effect was much less pronounced than with the water sample.

Human Lymphocyte Assays

In addition to the experiments with CHO cells, concentrated drinking water extracts have also been tested for clastogenic activity in human lymphocytes. The protocol used for the lymphocyte studies was similar to that which gave positive results in CHO cells (i.e., treatment in serum-free medium). In the first study, a concentrated extract (X030) was tested at doses of 1, 2, and 3 L/mL; at higher concentrations, the chromosomes were short and fuzzy and impossible to score. This extract had already been shown to give positive results in CHO cells (over a dose range of 1–3 L/mL) and in bacterial fluctuation assays. Untreated control, procedural blank, and positive control groups (cyclophosphamide, 6μg/mL) were also included in the experiment. The test was performed in the presence and absence of Aroclor-induced rat liver S9 and all treatments were carried out in duplicate. In this experiment, X030 showed no evidence of clastogenic activity in lymphocytes in either the presence or absence of S9 (see Table 6), although at 3 L/mL a significant reduction in the mitotic index was observed. Cyclophosphamide, the positive control, produced a clear increase in aberrations in the presence of S9.

The negative results in lymphocytes were somewhat unexpected in view of the potent clastogenic activity of such samples in CHO cells. A repeat experiment was therefore carried out using another drinking water extract (X055) prepared by a slightly different method. This method involved omitting the rotary evaporation

Table 4. Percentage of CHO cells with aberrations following treatment with extracts prepared from raw and final drinking water.

	Cells with aberrations,%[a]			
	Lowland water (X036)		Upland water (X071)	
Dose of extract, L/mL	Raw	Final	Raw	Final
4.0	—	—	18 (10)	—
3.0	—	—	12 (9)	89 (81)
2.5	—	—	—	58 (51)
2.0	18.6 (8.6)	94 (91)	16 (6)	17 (8)
1.75	17.4 (13)	93 (91)	—	—
1.5	14 (7)	68 (62)	—	8 (5)
1.0	11 (8)	23 (13)	12 (6)	10 (4)
0.5	7 (5)	7 (3)	—	—
Control	6 (4)		13 (3)	
Blank	9 (4)		8 (5)	
MNNG (1 μg/mL)	92 (90)		93 (91)	

[a] Figures in parentheses exclude cells with gaps only.

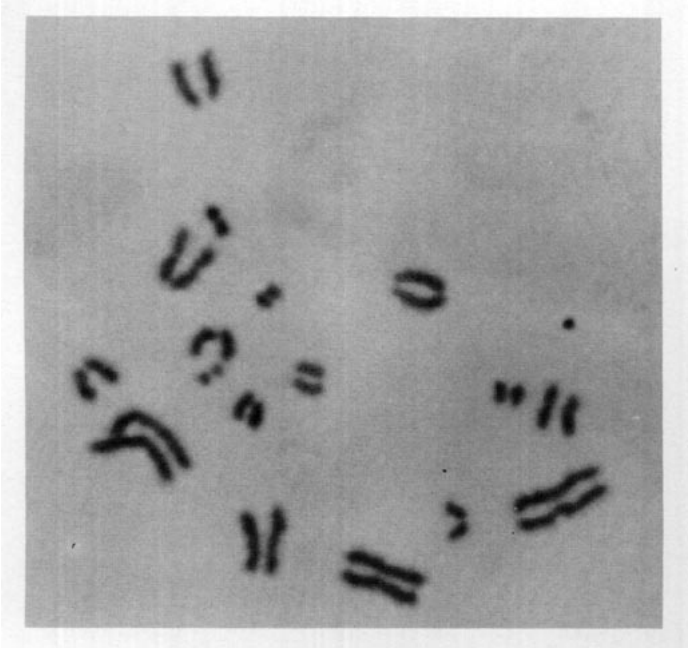

FIGURE 3. CHO cell treated with a concentrated raw water sample (2 L/mL) from a lowland river illustrating the effect on chromosome condensation and chromatid separation.

stage and evaporating the sample completely under nitrogen. Extracts prepared this way were found to be more potent in terms of mutagenic activity but, unfortunately, were also more toxic to the cells, and this extract could only be tested up to a maximum dose of 1 L/mL. The results obtained with X055 in lymphocytes are summarized in Table 7. Mitomycin C was used as the positive control in this experiment at concentrations of 0.5, 1.0, and 2.0 μg/mL. Some evidence of weak activity with X055 was apparent at the highest dose (1 L/mL) with a small increase in chromatid and chromosome breaks. However, in comparison with the clastogenic activity observed with such samples in CHO cells, the response was minimal.

A possible explanation for the poor response observed in lymphocytes could be protein binding of the active compounds. Although the cells were exposed to the extract in serum-free medium, whole-blood cultures contain many other cells as well as lymphocytes, in particular a large number of nondividing erythrocytes. It seemed possible that the mutagens in the extracts could bind to surface proteins on red blood cells, thus lowering the concentration available to react with lymphocytes. We decided, therefore, to carry out experiments using separated lymphocyte cultures.

Lymphocytes were separated from whole blood by centrifuging in a Ficoll-based medium and used in the cytogenetics assay (see "Materials and Methods"). When extracts prepared from lowland drinking waters were tested using this protocol, an obvious increase in chromosome aberrations, particularly chromatid breaks, was observed. However, treatment with these extracts produced short, fuzzy chromosomes that were difficult to score.

Recently, we have carried out a study with separated lymphocytes using a final water extract from an upland source (X071). The sample was tested at 2.0, 3.0, 3.5, and 4.0 L/mL, but, unfortunately, toxicity was observed at all but the lowest dose. At this dose, however, 39% of the cells contained aberrations; these aberrations were primarily chromatid breaks, but a few interchanges were recorded (see Table 8). It appears, therefore, that if separated lymphocytes are used, concentrated drinking water extracts can be shown to induce chromosome aberrations in human cells *in vitro*. Further studies are in progress to obtain dose-response data using this method.

In Vivo Study

In addition to the *in vitro* studies, a concentrated drinking water extract has also been tested for clastogenic activity in an *in vivo* mouse bone marrow cytogenetic assay. The extract used for this study (X021) has already been shown to give a positive result in CHO cells (see Table 3) and bacteria *in vitro*. Groups of mice were orally administered the extract at concentrations equivalent in mg/kg to 1, 3, and 10 years of consumption by the average human. Metaphase preparations were obtained from bone marrow after 6 hr to detect short-lived, rapidly formed lesions, after 24 hr (normally the most likely time interval for chromosome breakage to

Table 5. Percentages of CHO cells with aberrations following treatment with a final drinking water extract (X036) in the presence of different levels of serum.

Treatment	Serum in treatment medium, %	Number of metaphases analyzed	Cells with aberrations, %[a]		
			Chromatid	Chromosome	Total
X036 1.75 L/mL	0%	100	82 (79)	38 (38)	86 (83)
X036 1.75 L/mL	2%	100	58 (49)	23 (22)	66 (62)
X036 1.75 L/mL	5%	100	37 (23)	10 (10)	44 (30)
X036 1.75 L/mL	10%	100	19 (17)	6 (6)	24 (23)
X036 1.75 L/mL	15%	100	22 (17)	4 (3)	24 (19)
MNNG 1 μg/mL	0%	100	91 (90)	65 (63)	96 (95)
MNNG 1 μg/mL	15%	100	60 (53)	37 (35)	70 (66)
Untreated control	0%	100	9 (2)	2 (2)	10 (3)
Untreated control	15%	100	11 (2)	2 (1)	13 (4)

[a] Figures in parentheses exclude cells with gaps only.

Table 6. Percentage of cells with aberrations in human lymphocytes treated with a final drinking water extract (X030).

Treatment	Cells containing aberrations, %	
	− S9	+ S9
X030 3 L/mL	4, 7[a]	2, 2
X030 2 L/mL	7, 5	3, 6
X030 1 L/mL	6, 6	0, 2
Blank 3 L/mL	3, 3	3, 2
Control	2.2, 7	3, 6
Cyclophosphamide (6 μg/mL)	2, 3	39, 34

[a] Results with the two duplicate cultures for each treatment group.

be detected), and after 48 hr for persistent lesions or those requiring a long time to develop. No significant increase in chromosome breakage was observed in metaphase cells associated with any of the doses of water extract tested, whereas the positive control (Mitomycin C) showed clear clastogenic activity. Data from the 24-hr sampling time is summarized in Table 9. The water sample was thus considered to be negative in this test system.

Discussion

The experiments described in this paper demonstrate conclusively that extracts prepared from chlorinated, surface-derived drinking waters are capable of inducing chromosome damage in mammalian cells *in vitro*. In CHO cells, positive results have been obtained with both lowland and upland waters, and the major portion of activity has been shown to be produced when the water is chlorinated. The potency of the samples in the CHO assay was somewhat greater than we would have predicted on the basis of the activity of the samples in bacteria, with dose levels of 1 to 2 L/mL producing chromosome damage in most of the metaphase cells examined. Apart from this, the results obtained in mammalian cells appear to be consistent with conclusions we have formulated on the basis of bacterial mutagenicity assays.

One new observation with the mammalian cell assays was that the presence of serum in the treatment medium markedly reduced the clastogenic activity of the extracts. This finding presumably indicates that the mutagenic species bind readily to protein, which may have important implications for the transport and distribution of the mutagens around the body once they are ingested.

Table 7. Percentage of human lymphocytes with aberrations following treatment with a final drinking water extract (X055) and Mitomycin C.

Treatment	Number of metaphases analyzed	Cell with aberrations, %[a]		
		Chromatid	Chromosome	Total
X055 1.0 L/mL	100	4 (3)	6 (5)	10 (8)
X055 0.5 L/mL	100	5 (4)	0 (0)	5 (4)
X055 0.25 L/mL	100	5 (3)	0 (0)	5 (3)
Mitomycin C 0.5 μg/L	100	44 (39)	34 (33)	63 (57)
Mitomycin C 1.0 μg/L	100	29 (33)	13 (13)	61 (56)
Mitomycin C 2.0 μg/L	100	59 (55)	39 (37)	78 (74)
Untreated control	100	4 (0)	1 (1)	5 (1)

[a] Figures in parentheses exclude cells with gaps only.

Anthanasiou and Kyrtopoulos have also reported that drinking water extracts produced chromosome damage in CHO cells *in vitro* (*14*), but the doses needed to give a positive effect (i.e., around 400 L/mL) were much higher than those used in the present experiments. In their studies, however, treatment was carried out in media containing 10% FCS, which we have shown suppresses the activity of the extracts. Another possibility for the difference in results is that their method of producing concentrated extracts may have been less efficient than ours. Positive results with concentrated water samples in CHO cells have also been reported by Van Kriejl et al. (*15*), but this publication is an abstract of a meeting presentation, and few details are given.

To confirm the clastogenic activity of drinking water extracts, further studies were undertaken using human lymphocyte cultures. To observe a clear, positive effect, separated lymphocytes, free from red blood cells had to be used. The inhibition of activity by erythrocytes may be a simple protein-binding effect such as that observed with serum, although we cannot rule out the possibility of enzymatic detoxification of the mutagens. At a recent European Environmental Mutagen Society meeting (Stockholm, June 1985), Howard and Richardson presented data that showed that hydrogen peroxide was clastogenic in separated lymphocytes but not if whole-blood cultures were used. They attributed this effect to the presence of catalase and glutathione peroxidase in red blood cells that could detoxify the hydrogen peroxide.

In view of the results obtained *in vitro*, the absence of an effect in the mouse bone marrow study was not unexpected. The most likely explanation for the negative response is that following oral ingestion, the mutagenic compounds failed to reach the bone marrow. The failure to observe an effect in bone marrow cells does not prove that concentrated drinking water extracts are nongenotoxic *in vivo*. Even if the mutagens are not readily adsorbed, it is still possible that they could induce genetic change in the cells first encountered in the body. As such, cells in the esophagus, stomach, and intestinal tract are probably the most likely target sites for the type of mutagens that are produced during the chlorination of surface water. The importance of the gastrointestinal tract with respect to carcinogens in drinking water has also been implicated in several epidemiology studies (*16–18*).

For further *in vivo* studies, it would be useful to have a short-term assay in which animals could be orally administered concentrated water extracts and then genetic damage monitored in gastrointestinal cells. Unfortunately, at present, no well-validated method fulfills these requirements. However, over the last few years, interest in gastrointestinal carcinogens has been increasing and a number of laboratories are developing assays for their identification. These assays include the

Table 8. Frequency and type of chromosome damage observed in separated human lymphocyte cultures treated with a final drinking water extract (X071).[a]

Dose of extract, L/mL	Cells with aberrations, %[b]			Total aberrations per 100 cells						
				Chromatid				Chromosome		
	Chromatid	Chromosome	Total	G	B	I	Fr	G	B	Af
4.0		Toxic								
3.5		Toxic								
3.0		Toxic								
2.0	37 (33)	2 (2)	39 (35)	7	53	5	1	0	0	2
Blank (4.0 L/mL)	4 (2)	0 (0)	4 (2)	2	1	0	1	0	0	0
Control	3 (1)	0 (0)	3 (1)	2	1	0	0	0	0	0
Mitomycin C (2 μg/mL)	25 (22)	4 (3)	29 (25)	6	18	7	1	1	1	2

[a] G, gaps; B, breaks; I, interchanges; Fr, fragments (single); AF, acentric fragments.
[b] Figures in parentheses exclude cells with gaps only.

detection of nuclear anomalies in sections of colon tissue (*19–22*), sister-chromatid exchange in intestinal cells (*23–26*), and unscheduled DNA repair synthesis in stomach mucosa (*27*). It is hoped that one of these methods may prove suitable for evaluating the mutagenic potential of concentrated drinking water extracts *in vivo*.

Concentrated extracts prepared from chlorinated, surface-derived waters have now been shown to possess genotoxic activity in a wide range of *in vitro* assays using bacterial, rodent, and human cells. On the basis of these results, one must conclude that the chlorination of water results in the generation of compounds that represent a qualitative carcinogenic risk to consumers.

In vitro assays, although useful for identifying potential hazards, cannot be used to quantify the magnitude of the risk. Risk analysis relies either on extrapolation from animal experiments or on epidemiological studies and ideally requires accurate information on exposure levels. Both approaches, however, are relatively insensitive and are not particularly reliable for evaluating low-dose exposures that are likely to represent a low risk of disease. Furthermore, because we do not know the identity of the mutagens produced by chlorination, we cannot define the level of exposure. It should be recognized that, for some time, it will be difficult or impossible to estimate accurately the risk associated with consumption of chlorinated drinking water.

Most scientists working in this field would accept that if a carcinogenic hazard to consumers is associated with the use of chlorine for drinking water disinfection, the risk is likely to be small and is probably outweighed by the obvious benefits in controlling waterborne diseases. Unfortunately, we cannot rely on the public to take rational views on matters relating to cancer. It is likely that, if we are unable to define the risk accurately, increasing pressure (both public and political) will be exerted to encourage action to be taken on the basis of the results obtained in *in vitro* assays, with the view that even a potential carcinogenic risk from drinking water is unacceptable. Providing that the cost is not prohibitive, some water utilities may decide that any remedial action that will pre-empt a public outcry can be justified on the basis of public relations.

It is important, therefore, to obtain information on

Table 9. Summary of chromosome damage observed in mouse/bone marrow cells following treatment with a concentrated drinking water extract (24 hr sampling time).

Treatment	Sex	Cells with aberrations, %[a]	Total numbers of aberrations					
			Chromatid			Chromosome		
			Gaps	Del.	Int.	Af	Dic.	Others[b]
Negative control	M	1.6 (0.4)	6	3	0	0	0	0
	F	2.0 (0.6)	7	3	0	0	0	0
Procedural blank	M	2.4 (1.4)	6	7	0	0	0	0
	F	2.6 (0.8)	9	3	1	0	0	0
1 Year	M	1.6 (0.6)	5	3	0	0	0	0
	F	1.0 (0.6)	2	3	0	0	0	0
3 Years	M	2.4 (0.6)	10	2	0	1	0	0
	F	2.4 (1.0)	7	5	0	0	0	0
10 Years	M	1.8 (0.4)	7	2	0	0	0	0
	F	2.6 (1.2)	7	5	1	0	0	0
Mitomycin C	M	23.4 (19.6)	30	110	14	6	1	90
	F	16.8 (14.8)	26	62	20	3	1	30

[a] Figures in parentheses exclude cells with gaps only.
[b] Multiple aberrations (i.e., > 10 aberrations/cell, counted as 10).

possible ways to remove mutagens from water or to prevent their formation. Filtration through granular activated carbon (GAC) beds appears to be the most promising method for removing mutagens (*28, 29*), although questions, such as optimum flow rates through the beds, contact period with the carbon, and carbon regeneration times, need to be considered. GAC filtration may also offer other benefits, such as taste and odor control and an overall better quality of water, which may make it an attractive option for some water utilities. The use of alternative disinfectants, such as ozone, chlorine dioxide, and chloramines, should also be considered. However, their use may present other toxicological problems that need to be fully evaluated before any recommendation can be made (*30–32*). Even if alternative treatments are rejected, the questions raised concerning the long-term health effects of chlorinated organics in drinking water may result in a more judicious use of chlorine as a disinfectant.

This work was funded by the Department of the Environment to whom we are grateful for permission to publish.

The *in vivo* mouse bone marrow study was carried out at University College, Swansea, by Dr. N. Danford and Dr. J. M. Parry under contract to the Water Research Centre. The initial study using human lymphocytes was subcontracted to Life Science Research Laboratories.

The work described in this paper was not funded by the U.S. Environmental Protection Agency, and no official endorsement should be inferred.

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