WATER RESEARCH

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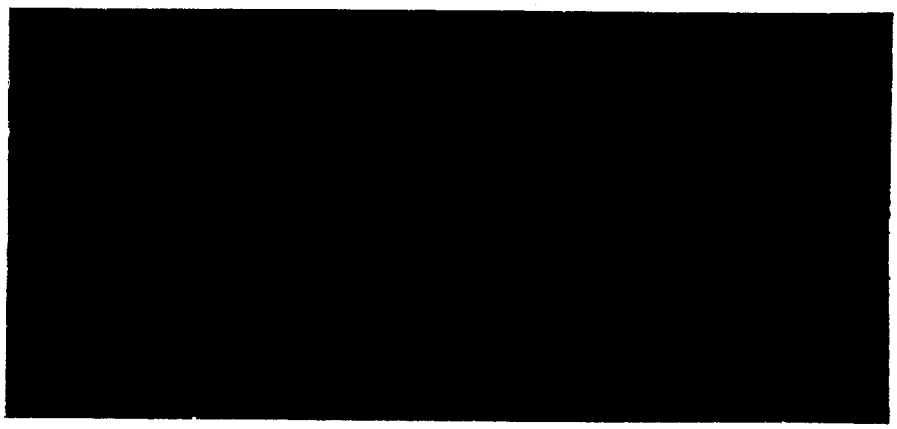

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January 1987

DoE 1442-M

REPORT OF RESEARCH UNDERTAKEN AT LIFE SCIENCE RESEARCH TO DEVELOP A METHOD FOR TESTING CONCENTRATED WATER EXTRACTS IN SEPARATED HUMAN LYMPHOCYTE CULTURES (EHT 9154 SLD)

DoE Contract Reference: PECD 7/7/94-94/83
Contract Duration: August 1983 - July 1986

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PREFACE

The work described in this report was carried out as part of a contract with the Department of the Environment (Ref PECD 7/7/94-94/83) to evaluate the use of biological screening tests for the assessment of drinking water quality.

Studies conducted at WRc had demonstrated that concentrated drinking water extracts produce chromosome damage in Chinese hamster ovary (CHO) cells treated in vitro. As a follow-up to this work, a sub-contract was placed with Life Science Research (LSR) to evaluate the clastogenic activity of a concentrated water extract (that had already been shown to be active in CHO cells) in cultured human lymphocytes. In this study, which used whole blood cultures, the extract showed no evidence of activity. Preliminary investigations indicated that the presence of red blood cells in the culture may have suppressed the clastogenicity of the extract to the lymphocytes. A second contract was therefore placed with LSR to develop and optimise a method for testing water extracts for clastogenic activity using separated lymphocyte cultures. This report describes a series of experiments that were carried out by LSR on this technique.

86/WRC003/702

DEVELOPMENTAL PROJECT REPORT

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DEVELOPMENTAL PROJECT REPORT

LSR Report No. : 86/WRC003/702

We, the undersigned, hereby declare that the report following constitutes a true and faithful account of the procedures adopted, and the results obtained, in the performance of this Study.

The work reported here was conducted so as to comply with current Good Laboratory Practice regulations. However, due to its essentially developmental nature, and as it lead to a definitive Study which is to be reported elsewhere, this report has not been reviewed by the LSR Quality Assurance Unit.

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1. SUMMARY

Ten separate experiments were performed, involving method evaluation and validation, and also including cytogenetic assays on two concentrated water samples, X055 and X098.

Initial method evaluation led to the selection of RPMI 1640 as a suitable growth medium for separated human peripheral lymphocytes. Further experiments revealed that total serum deprivation for periods up to and including four hours in no way affected survival of separated human lymphocytes, and that addition of donor serum to cultures during both the establishment and post-treatment periods was beneficial to cell growth. A 72 hour pre-treatment establishment period was selected in order to ensure that sufficient cells had accumulated prior to treatment. Therefore, cultures were established in RPMI 1640 complete culture medium containing 5% donor serum and incubated at 37°C for 72 hours prior to treatment. Treatment was for a period of three hours in serum-free medium and, following treatment, cultures were re-established in complete culture medium (with 5% donor serum) for a 21 hour post-treatment recovery period.

Investigation of the known direct-acting clastogens chlorambucil and ethyl methanesulphonate resulted in the selection of a chlorambucil concentration of 2.5ug/ml for use as the positive control in separated lymphocyte cultures.

Toxicity and cytogenetic assays on X055 showed gross toxicity at concentrations of 2.0 and 1.5 litres/ml (no metaphases present for analysis), and at 1.0 litre/ml 24% of metaphases seen contained damaged chromosomes. This sample was considered unduly toxic by the Study Sponsor, and further testing was peformed on a replacement sample, X098.

Toxicity and cytogenetic assays showed that X098 showed some limited evidence of clastogenic potential at both 1.0 and 2.0 litres/ml when gap-type aberrations were included in the total aberrations seen. However, no real increase in damage other than gaps was seen over control values at any X098 concentration.

Consideration of mitotic indices showed a dose-related cytotoxic effect in response to X098 treatment, with reductions in mitotic index (compared to the control value) of 52, 84, 95 and 100% at concentrations of 0.5, 1.0, 2.0 and 3.0 litres/ml respectively.

As a result of these procedural evaluation assays, a definitive study has been initiated; this will be fully described in a future report.

2. TEST MATERIALS

2.1 SAMPLE X055

This sample was received from the Study Sponsor during January 1985, and was stored deep-frozen at -20°C until required.

Sample preparation
(information supplied by the Study Sponsor)

150 litres of water was passed through an XAD-2 column, the column eluted with acetone, and the eluate transferred to 15ml of sterile, purified water. The redissolved eluate was then filter sterilised through a 0.45 um membrane filter.

The concentration factor of the sample was 1×10^4, i.e. 1 litre of original water yielded 100ul of extract.

2.2 SAMPLE X098

This sample (totalling 15ml in volume) was received from the Study Sponsor on 17 December 1985, and was stored deep-frozen at -20°C until required.

Sample preparation
(information supplied by the Study Sponsor)

450 litres of water was passed through XAD-2 columns at a flow rate of 100 ml/minute. The columns were then blown dry with nitrogen and eluted with acetone. The acetone eluate was taken to dryness, and the residual material redissolved in 45ml of deionised/activated carbon filtered water. The sample was then filter sterilised through a 0.45 um membrane filter.

The overall concentration factor of the sample was 1×10^4, i.e. 1 litre of original water yielded 100ul of extract.

2.3 DILUTION WATER

In addition, a sample of deionised water passed through activated carbon, and then autoclaved, was also supplied by the Study Sponsor during January 1985. This sample was to be used for preparing dilutions of the X055 and X098 samples for addition to test cultures, and also for use in negative control cultures.

3. EXPERIMENTAL DESIGN, RESULTS AND DISCUSSION

3.1 EXPERIMENT 1

Date : March 1985

Objective : To investigate comparative growth of separated lymphocytes in Hams' F10 and RPMI 1640 medium.

Design : Cultures of separated human peripheral lymphocytes were established in both Hams' F10 and RPMI 1640 complete medium (see Appendices 1 and 2). Cultures were incubated for a total of 72 hours at 37°C, with no "treatment period", and the cells harvested (see Appendix 2). Slides were prepared and stained, and cell proliferation assessed by low power (x10 objective) microscopic examination (see Appendix 2).

Results : Higher mitotic indices and greater cell proliferation were seen in cultures grown in RPMI 1640 complete culture medium.

Conclusion : RPMI 1640 medium was selected for culture establishment and treatment in future experiments.

3.2 EXPERIMENT 2

Date : April 1985

Objective : To compare the effects of a two- or four-hour treatment period in serum-free RPMI 1640 treatment medium.

Design : Cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete culture medium. After 48 hours of incubation at 37°C, cultures were resuspended in 4.5 ml serum-free treatment medium (see Appendix 1), and incubated in a shaking water bath at 37°C for 2 or 4 hours. Cultures were then resuspended in complete culture medium to a final volume of 10 ml, and incubated at 37°C for a further 22 or 20 hours respectively. After this time, cells were harvested, and slides prepared and stained. Mitotic index was scored for each culture (see Appendix 2).

Results :

Treatment time (hours)	Mitotic Index	Mean Mitotic Index
2	4.0	4.05
2	4.1	
4	2.2	3.30
4	4.4	

Conclusion : Mitotic activity did not appear to be adversely affected by serum deprivation for up to 4 hours. A four-hour treatment period could therefore be used in future assays.

3.3 EXPERIMENT 3

Date : April 1985

Objective : Initial cytogenetic assay on X055 concentrated water sample.

Design : Cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium and incubated at 37°C for 48 hours.

Treatment schedule

Culture No.	Treatment
1	Dilution water
2	X055 : 0.5 litres/ml
3	X055 : 1.0 litres/ml
4	X055 : 1.5 litres/ml
5	X055 : 2.0 litres/ml

Test solutions were prepared as follows on the day of treatment, using the dilution water supplied.

Culture No.	Volume X055 (ml)	Volume dilution water (ml)
1	0.00	2.00
2	0.25	1.75
3	0.50	1.50
4	0.75	1.25
5	1.00	1.00

Test solutions were added to 0.5ml single-strength treatment medium, 2.0ml double-strength treatment medium (see Appendix 1) and 0.5ml cell pellet, giving a total culture volume of 5ml. Cultures were incubated in a shaking water bath at 37°C for 4 hours. Cultures were then resuspended in complete culture medium to a final volume of 10ml, and incubated at 37°C for a further 20 hours. After this time, cells were harvested, and slides prepared and stained. Up to 100 metaphases from each culture were then examined for chromosomal damage (see Appendix 2).

3.3 **EXPERIMENT 3** (continued)

Results :

Culture No.		1	2	3	4	5
Number of cells scored		25	100	41[b]	a	a
% cells aberrant		0	5	24.4		
% cells aberrant excluding gaps		0	2	12.2		
Number and specific types of chromosomal aberration	SSG		3	1		
	DSG			1		
	SSB					
	DSB					
	E			1		
	F		2	1		

SSG - Single-strand gap SSB - Single-strand break
DSG - Double-strand gap DSB - Double-strand break
E - Exchange F - Fragment
a - Few cells and no metaphases present ; not scored.
b - Remaining metaphases too fuzzy for analysis.

Conclusion : Following consideration of the findings of this initial cytogenetic assay, the X055 concentrations selected for use in a main cytogenetic assay were 0.50, 0.75, 1.00 and 1.25 litres/ml.

Information from the Study Sponsor indicated that X055 was unduly toxic; it was therefore decided that a replacement sample, X098, would be provided. In addition, it was agreed that the treatment period was to be reduced to three hours in all subsequent studies.

3.4 EXPERIMENT 4

Date : January 1986

Objective : Comparison of two direct-acting clastogens for suitability as positive control treatments in separated lymphocyte assays.

Design : Cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium and incubated at 37°C for 48 hours.

Treatment schedule

Culture No.	Treatment	Concentration Initial (mg/ml)	Concentration Final (ug/ml)
1	Ethanol	-	-
2	Chlorambucil	0.5	2.5
3	Chlorambucil	1.0	5.0
4	Chlorambucil	1.5	7.5
5	dH_2O	-	-
6	EMS	20.0	100.0
7	EMS	40.0	200.0
8	EMS	60.0	300.0

Test solutions of chlorambucil (in ethanol) and ethyl methanesulphonate (EMS: in sterile, distilled water) were prepared on the day of treatment, and 25ul of control/test solution added to each 5ml culture (in serum-free treatment medium) in accordance with the treatment schedule. Cultures were then incubated in a shaking water bath at 37°C for 3 hours. Following this treatment, cultures were resuspended in complete culture medium to a final volume of 10ml, and incubated at 37°C for a further 21 hours. After this time, cells were harvested, and slides prepared and stained. Up to 50 metaphases from each culture were then examined for chromosomal damage.

Results :

Culture No.	No. cells scored	Percent aberrant metaphases With gaps	Percent aberrant metaphases Without gaps
1	50	4.0	0.0
2	6	16.7	16.7
3	No analysable metaphases seen		
4	1	100.0	100.0
5	50	4.0	2.0
6	50	8.0	4.0
7	50	2.0	0.0
8	8	0.0	0.0

Conclusion : Following consideration of the findings of this assay, further work to select a direct-acting clastogen was restricted to use of chlorambucil alone.

3.5 EXPERIMENT 5

Date : January 1986

Objective : Selection of a chlorambucil concentration for use in positive control cultures in separated lymphocyte cytogenetic assays.

Design : Cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium and incubated at 37°C for 48 hours.

Treatment schedule

Culture No.	Treatment	Concentration Initial (mg/ml)	Concentration Final (ug/ml)
9	Ethanol	-	-
10	Chlorambucil	0.2	1.0
11	Chlorambucil	0.3	1.5
12	Chlorambucil	0.4	2.0
13	Chlorambucil	0.5	2.5
14	Chlorambucil	0.6	3.0
15	Chlorambucil	0.7	3.5

Test solutions of chlorambucil (in ethanol) were prepared on the day of treatment, and 25ul of control/test solution added to each 5ml culture (in serum-free treatment medium) in accordance with the treatment schedule. Cultures were then incubated in a shaking water bath at 37°C for 3 hours. Following this treatment, cultures were resuspended in complete culture medium to a final volume of 10ml, and incubated at 37°C for a further 21 hours. After this time, cells were harvested, and slides prepared and stained. Up to 50 metaphases from each culture were then examined for chromosomal damage.

Results :

Culture No.	No. cells scored	Percent aberrant metaphases With gaps	Percent aberrant metaphases Without gaps
9	50	6.0	0.0
10	50	20.0	8.0
11	50	28.0	14.0
12	50	42.0	30.0
13	50	30.0	24.0
14	29	48.3	31.0
15	16	81.3	50.0

Conclusion : Following consideration of the findings of this assay, a chlorambucil concentration of 2.5ug/ml was selected for use in positive control cultures of separated lymphocyte assays.

Following discussion with the Study Sponsor, a further experiment was conducted to assess the effect of an extended post-treatment recovery period on the survival of damaged cells following treatment with chlorambucil concentrations up to 7.5ug/ml.

3.6 EXPERIMENT 6

Date : February 1986

Objective : Determination of the effect of an extended post-treatment recovery time for cultures of separated lymphocytes treated with chlorambucil.

Design : Cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium and incubated at 37°C for 48 hours.

Treatment schedule

Culture No.	Treatment	Concentration Initial (mg/ml)	Concentration Final (ug/ml)
16	Ethanol	-	-
17	Chlorambucil	0.5	2.5
18	Chlorambucil	1.0	5.0
19	Chlorambucil	1.5	7.5

Test solutions of chlorambucil (in ethanol) were prepared on the day of treatment, and 25ul of control/test solution added to each 5ml culture (in serum-free treatment medium) in accordance with the treatment schedule. Cultures were then incubated in a shaking water bath at 37°C for 3 hours. Following this treatment, cultures were resuspended in complete culture medium to a final volume of 10ml, and incubated at 37°C for a further 45 hours. After this time, cells were harvested, and slides prepared, stained and examined.

Results : Cell growth was seen to be very poor throughout all cultures; when slide preparations were examined, only one analysable metaphase was seen.

Conclusion : Following consideration of the findings of this assay, a chlorambucil concentration of 2.5ug/ml was selected for use in positive control cultures of separated lymphocyte assays, with a post-treatment recovery period of 21 hours.

3.7 EXPERIMENT 7

Date : February 1986

Objective : Initial cytogenetic assay on X098 concentrated water sample.

Design : Six cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium and incubated at 37°C for 48 hours.

Treatment schedule

Culture No.	Treatment
1	Dilution water
2	X098 : 1.0 litres/ml
3	X098 : 2.0 litres/ml
4	X098 : 3.0 litres/ml
5	X098 : 4.0 litres/ml
6	Chlorambucil : 2.5 ug/ml

Test solutions were prepared as follows on the day of treatment, using the dilution water supplied.

Culture No.	Volume X098 (ml)	Volume dilution water (ml)
1	0.0	2.0
2	0.5	1.5
3	1.0	1.0
4	1.5	0.5
5	2.0	0.0

Test solutions were added to 0.5ml single-strength treatment medium, 2.0ml double-strength treatment medium and 0.5ml cell pellet, giving a total culture volume of 5ml. Cultures were incubated in a shaking water bath at 37°C for 3 hours. Cultures were then resuspended in complete culture medium to a final volume of 10ml, and incubated at 37°C for a further 21 hours. After this time, cells were harvested, and slides prepared, stained and examined.

Results : Poor cell growth was seen throughout; when slide preparations were examined, no analysable metaphases were seen.

Conclusion : Following consideration of the findings of this assay it was decided, in consultataion with the Study Sponsor, to re-evaluate the methodology by assessing the effects of incorporation of donor serum (at a concentration of 5%) into cultures of separated lymphocytes.

3.8 EXPERIMENT 8

Date : February 1986

Objective : To investigate the effects of donor serum addition on the growth and survival of separated human peripheral lymphocytes.

Design : Cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium as per the method detailed in Appendix 2, but with the addition of 5% donor serum (i.e. cultures comprised 0.5ml cell pellet, 0.5ml phytohaemagglutinin, 8.5ml complete culture medium and 0.5ml donor serum). After 48 hours of incubation at 37°C, cultures were resuspended in serum-free treatment medium to a final volume of 5ml, and incubated in a shaking water bath at 37°C for 3 hours. Cultures were then resuspended in complete culture medium, again with the addition of 5% donor serum, to a final volume of 10ml and incubated at 37°C for a further 21 hours. After this time, cells were harvested, and slides prepared, stained and examined.

Results : It was judged that sufficient metaphases were present for chromosomal analysis.

Conclusion : Addition of 5% donor serum apparently resulted in improved cell proliferation. Donor serum inclusion was incorporated into the experimental procedure for all future assays.

3.9 EXPERIMENT 9

Date : March 1986

Objective : Second cytogenetic assay on X098 concentrated water sample.

Design : Five cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium, with the addition of 5% donor serum, and incubated at 37°C for 48 hours.

Treatment schedule

Culture No.	Treatment
1	Dilution water
2	X098 : 0.5 litres/ml
3	X098 : 1.0 litres/ml
4	X098 : 2.0 litres/ml
5	X098 : 3.0 litres/ml

Test solutions were prepared as follows on the day of treatment, using the dilution water supplied.

Culture No.	Volume X098 (ml)	Volume dilution water (ml)
1	0.00	2.00
2	0.25	1.75
3	0.50	1.50
4	1.00	1.00
5	1.50	0.50

Test solutions were added to 0.5ml single-strength treatment medium, 2.0ml double-strength treatment medium and 0.5ml cell pellet, giving a total culture volume of 5ml. Cultures were incubated in a shaking water bath at 37°C for 3 hours. Cultures were then resuspended in complete culture medium (with 5% donor serum) to a final volume of 10ml and incubated at 37°C for a further 21 hours. After this time, cells were harvested, and slides prepared, stained and examined.

Results : Insufficient cells were available for chromosomal evaluation.

Conclusion : Following consideration of the findings of this assay it was decided, in consultation with the Study Sponsor, to re-evaluate the methodology by assessing the effects of a 72 hour pre-treatment establishment period on cultures of separated lymphocytes.

3.10 EXPERIMENT 10

Date : June 1986

Objective : Third cytogenetic assay on X098 concentrated water sample, incorporating a 72 hour pre-treatment culture establishment phase.

Design : Five cultures of separated human peripheral lymphocytes were established in RPMI 1640 complete medium, with the addition of 5% donor serum, and incubated at 37°C for 72 hours.

Treatment schedule

Culture No.	Treatment
1	Dilution water
2	X098 : 0.5 litres/ml
3	X098 : 1.0 litres/ml
4	X098 : 2.0 litres/ml
5	X098 : 3.0 litres/ml

Test solutions were prepared as follows on the day of treatment, using the dilution water supplied.

Culture No.	Volume X098 (ml)	Volume dilution water (ml)
1	0.00	2.00
2	0.25	1.75
3	0.50	1.50
4	1.00	1.00
5	1.50	0.50

Test solutions were added to 0.5ml single-strength treatment medium, 2.0ml double-strength treatment medium and 0.5ml cell pellet, giving a total culture volume of 5ml. Cultures were incubated in a shaking water bath at 37°C for 3 hours. Cultures were then resuspended in complete culture medium with (5% donor serum) to a final volume of 10ml and incubated at 37°C for a further 21 hours. After this time, cells were harvested, and slides prepared and stained. Mitotic indices were scored for each culture, and up to 50 metaphases examined from each culture for chromosomal aberrations.

3.10 **EXPERIMENT 10** (continued)

Results :

Culture No.		1	2	3	4	5
Mitotic Index		9.5	4.6	1.5	0.5	0.0
Number of cells scored		50	50	50	50	a
% cells aberrant		0	0	12	10	
% cells aberrant excluding gaps		0	0	6	6	
Number and specific types of chromosomal aberration	SSG			4	1	
	DSG				1	
	SSB			3		
	DSB					
	E					
	F			1	3	
	(En)	(6)	(1)	(2)		

SSG - Single-strand gap SSB - Single-strand break
DSG - Double-strand gap DSB - Double-strand break
E - Exchange F - Fragment
(En)- Endoreduplicated cells ; see below.
a - Few cells and no metaphases present ; not scored.

Endoreduplicated cells were also seen in all cultures, including the negative controls. However, as these do not represent a product of chromosome breakage, they have not been included in the results tabulated above.

Conclusion : From the findings of this assay it is seen that X098 showed some limited evidence of clastogenic potential at both 1.0 and 2.0 litres/ml when gap-type aberrations were included in the total aberrations seen. However, no real increase in damage other than gaps was seen over control values at any X098 concentration.

Consideration of mitotic indices showed a dose-related cytotoxic effect in response to X098 treatment, with reductions in mitotic index (compared to the control value) of 52, 84, 95 and 100% at concentrations of 0.5, 1.0, 2.0 and 3.0 litres/ml respectively.

Therefore it is concluded that, as the biological significance of gap-type aberrations is questionable, X098 showed no clear evidence of clastogenic potential even at toxic concentrations.

4. FUTURE WORK

Based on the findings of these developmental assays, a definitive experiment has been initiated in which concentrated water sample X111/116 will be tested, using the methodology described in Experiment 10 and incorporating a procedural blank, X077. This is to be fully described in a future report.

APPENDIX 1

MEDIA PREPARATION

Saline/Heparin (50iu/ml)

495 ml physiological saline (0.9%)
5 ml heparin solution (5000iu/ml)

EXPERIMENT 1

Hams' F10 complete culture medium

82.0 ml Hams' F10 medium (single-strength)
15.0 ml Foetal calf serum
1.6 ml 7.5% sodium bicarbonate solution
1.0 ml L-glutamine (200mM)
0.4 ml Penicillin/Streptomycin (5000iu/ml : 5000ug/ml)

RPMI 1640 complete culture medium

84.6 ml RPMI 1640 Dutch modification medium (single-strength), with sodium bicarbonate and L-glutamine
15.0 ml Foetal calf serum
0.4 ml Penicillin/Streptomycin (5000iu/ml : 5000ug/ml)

EXPERIMENTS 2, 4, 5, 6, and 8

Complete culture medium

Prepared from 10x RPMI 1640 medium diluted with sterile, distilled water.

Single-strength RPMI 1640 medium containing the following:
1.0% L-glutamine (200mM)
0.4% Penicillin/Streptomycin (5000iu/ml : 5000ug/ml)
2.7% 7.5% sodium bicarbonate solution
15.0% Foetal calf serum

Serum-free treatment medium

Prepared from 10x RPMI 1640 medium diluted with sterile, distilled water.

Single-strength RPMI 1640 medium containing the following:
1.0% L-glutamine (200mM)
2.7% 7.5% sodium bicarbonate solution

APPENDIX 1 (continued)

MEDIA PREPARATION

EXPERIMENTS 3, 7, 9 and 10

Complete culture medium

Prepared from 10x RPMI 1640 medium diluted with sterile, distilled water.

Single-strength RPMI 1640 medium containing the following:

1.0%	L-glutamine (200mM)
0.4%	Penicillin/Streptomycin (5000iu/ml : 5000ug/ml)
2.7%	7.5% sodium bicarbonate solution
15.0%	Foetal calf serum

Single-strength serum-free treatment medium

Prepared from 10x RPMI 1640 medium diluted with sterile, distilled water.

Single-strength RPMI 1640 medium containing the following:

1.0%	L-glutamine (200mM)
2.7%	7.5% sodium bicarbonate solution

Double-strength serum-free treatment medium

Prepared from 10x RPMI 1640 medium diluted with sterile, distilled water.

Double-strength RPMI 1640 medium containing the following:

2.0%	L-glutamine (200mM)
5.4%	7.5% sodium bicarbonate solution

APPENDIX 2

EXPERIMENTAL PROCEDURE

1. Establishment of separated lymphocyte cultures

Human peripheral blood is obtained by venepuncture from healthy, human, non-smoking, male volunteers not known to be taking any medication. Volumes of whole blood are collected into vessels containing an equal volume of saline/heparin solution (see Appendix 1) and thoroughly mixed by inversion. 5ml aliquots of blood mixture are then carefully layered onto 5ml aliquots of Histopaque (Sigma Chemicals) contained in sterile centrifuge tubes, and the tubes centrifuged at 3000 rpm for 20 minutes. Following centrifugation, the uppermost plasma layer is removed (and, in experiments 8, 9 and 10, this is retained deep-frozen for addition to test cultures); the underlying lymphocyte and saline/heparin layers are collected together and transferred to clean, sterile centrifuge tubes. The remaining blood pellet is discarded. Further saline/heparin is added to the lymphocytes, to a total volume of 10ml, and tubes are then centrifuged at 2000 rpm for 10 minutes. Following this, the supernatant is pipetted off and the cell pellet resuspended in 8 ml fresh saline/heparin. After further centrifugation at 1200 rpm for 10 minutes, each cell pellet is resuspended in sterile complete culture medium (containing 5% phytohaemagglutinin to stimulate cell division), and pairs of cell suspensions are combined to form single cultures to a final volume of 10ml. The cultures are incubated at 37°C for the required pre-treatment timespan, with occasional shaking to prevent cell clumping.

2. Culture treatment

After the required pre-treatment incubation period, cultures are centrifuged (1000 rpm, 5 minutes), the supernatant removed and the cell pellet resuspended in an appropriate volume of serum-free treatment medium in accordance with the precise experimental protocol for each individual experiment. Following the required treatment period, cultures are centrifuged, and the cell pellet resuspended in complete culture medium for the final post-treatment period.

3. Culture harvesting

Three hours prior to termination, cell division is arrested by the addition of the spindle poison, Colcemid, to a final concentration of 0.4 ug/ml. Following the three hour Colcemid treatment, cells are harvested by low-speed centrifugation, and the pellets of cells thus collected are resuspended in hypotonic (0.56%) potassium chloride solution, and later fixed in freshly prepared methanol : glacial acetic acid fixative (3:1 v/v).

APPENDIX 2 (continued)

EXPERIMENTAL PROCEDURE

4. Slide preparation

After at least three changes of fixative, metaphase chromosome spreads are prepared on clean, moist, grease-free glass slides. The slides are allowed to air-dry, and are then stained in Giemsa stain (1:9 in Sorensens' buffer), washed in buffer and allowed to dry. The slides are then cleared in xylene, and coverslips applied using DPX mountant.

5. Slide evaluation

5.1 Mitotic index

A measurement of gross toxicity to dividing lymphocytes is obtained by calculation of the mitotic index. This is based on the number of metaphases observed per 1000 cells scored, and is expressed as a percentage.

5.2 Metaphase analysis

Under high power (x100, oil immersion objective), metaphases are examined for chromosomal damage. Specific numbers and types of aberrations are recorded and these are grouped under the headings of "Total aberrations" or "Aberrations excluding gaps".

Chromosomal damage is recorded as follows:

Gap	: achromatic region in chromatid(s) no greater than the width of a chromatid: recorded as single strand (chromatid) or double strand (chromosome).
Break	: achromatic region in chromatid(s) greater than the width of a chromatid, or a discontinuity with displacement: recorded as single strand (chromatid) or double strand (chromosome).
Fragment	: any free, displaced portion of chromatid material.
Exchange	: aberration arising from an exchange between two or more chromosomes which results in the products reuniting to form a dicentric or polycentric structure.
Endoreduplication	: diplochromosomes, quadruple chromosomes (etc) in which 4, 8 (or more) chromatids are held together at the centromere.
Pulverised cells	: extreme fragmentation of chromosomal material.

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