Cytogenetic toxicity of D_2O in human lymphocyte cultures. Increased sensitivity in Fanconi's anemia[1]

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Summary. Chromosomal aberrations were scored in lymphocyte cultures from healthy individuals, patients with Bloom syndrome, and patients with Fanconi's anemia, after 4–5 h exposure to culture medium containing 90% heavy water (D_2O). D_2O treatment resulted in occasional pulverization of metaphases, and increased frequencies of chromosomal breakage. Patients with Fanconi's anemia were particularly sensitive to the chromosome breaking effect of D_2O.

Natural water contains only a trace amount (about 0.02%) of deuterium oxide (D_2O). Higher D_2O concentrations, up to 20%, can be tolerated by eukaryotic organisms, but still higher concentrations are generally cytotoxic[2,3]. We have used the human lymphocyte culture system to investigate whether short-term exposure to high levels of D_2O can have cytogenetic effects.

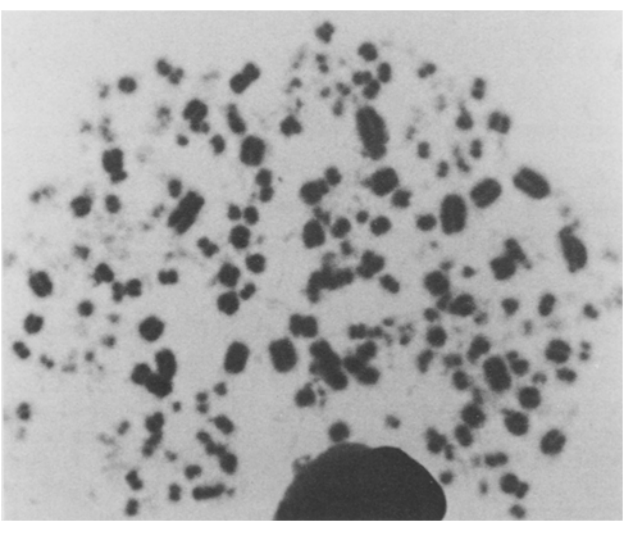
Materials and methods. Human lymphocyte cultures were initiated by adding 0.4–0.5 ml heparinized venous blood to 4.5 ml 'culture medium': Ham's F10 medium containing 15% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and phytohemagglutinin (type HA 15, Wellcome Research laboratories, Beckenham, England; 0.1 ml per culture). At 4–5 h before harvest some cultures were centrifuged (5 min, 160 × g) and their supernatants replaced by the above culture medium of which the Ham F10 and fetal calf serum ingredients (obtained in powder form from Flow Laboratories and Difco Laboratories, respectively) had been dissolved in either H_2O or D_2O (99.75% deuterium oxide, Merck); in the latter case, due to some dilution by the H_2O present in the cell pellet, the final D_2O concentration in the cell suspension was estimated to be $\geqslant 90\%$ (v/v); in other experiments (e.g., table 2) the serum components were omitted from the D_2O medium. After medium replacement incubation was continued in the dark at 37 °C until harvest, the last 60–90 min in the presence of colchicine (0.5 µg/ml). Cultures were harvested at 72 h and processed for chromosomal analysis as described[4]. Giemsa-stained metaphases were scored for chromosomal aberrations on coded slides; where possible, 100 cells were inspected per culture; chromatid gaps and chromatid breaks were distinguished as recommended[5].

Patients. The Bloom syndrome patients studied here were numbers 65–68 in the Bloom syndrome registry[6]; detailed clinical and cytogenetic data are reported by Hustinx et al.[7]. Diagnosis of the Fanconi's anemia patients was confirmed cytogenetically by their hypersensitivity to Mitomycin C[8].

Results. Various effects of exposure to D_2O were noted. Pilot experiments, in which the effect of long term exposures were tested, showed complete absence of mitoses after 72 h culture time in D_2O medium. 16-h exposure allowed very few divisions to appear, but the chromosomes were so condensed that they could not be scored for aberrations. Finally, an exposure time of 4–5 h usually yielded scorable metaphases, although in some cases the mitotic yields were still very low. The cytogenetic effect of 4–5 h exposure to D_2O medium was evaluated in lymphocyte cultures from healthy individuals, patients with Bloom syndrome and patients with Fanconi's anemia; in addition to a variety of other symptoms, these autosomal recessive 'chromosomal breakage syndromes' are characterized by increased frequencies of 'spontaneous' chromosomal aberrations and an increased cancer risk[9].

One feature of the 4–5 h exposed cultures was the presence of metaphases (about 25 in 2000 cells) with numerous chromosomal fragments, somewhat reminiscent of the pulverized metaphases resulting from premature chromosome condensation[10,11] (fig.). Occasionally, one or more 'pulverized' chromosomes were seen in a metaphase that was otherwise normal. There was no evidence for an increased incidence of these peculiar methaphases in the patients with chromosomal breakage syndromes. Evaluation of all scored metaphases revealed that the D_2O-treated cultures had increased frequencies of chromatid gaps, chromatid breaks and isochromatid breaks. Table 1 shows the incidence of cells with one or more of these particular aberrations; the frequency of other aberrations like chromatid interchanges, dicentrics and rings, which occur 'spontaneously' at elevated levels in the patients with chromosomal breakage, were not significantly affected by the D_2O treatment, and have therefore been ignored in the analysis summarized in table 1.

In all experiments there was an increased incidence of cytogenetically aberrant cells in the D_2O-treated cultures, although the effect was variable from experiment to experiment. Thus, in the group of healthy individuals the percentage of D_2O-induced aberrant cells (that is, after correction for the incidence rate in the untreated or H_2O medium control) varied from 1–38% (average 13.2%), in the Bloom syndrome patients from 4–27% (average 17%) and in the Fanconi's anemia group from 3–45% (average 30.5%). After D_2O treatment the mean numbers of breaks per aberrant cell for these groups were 1.3, 1.7 and 2.1, respectively. Both the mean percentage of D_2O-induced aberrant cells and the mean number of breaks per aberrant cell suggested that Fanconi's anemia patients may have an elevated sensitivity to the chromosome-breaking effect of D_2O. This suggestion was substantiated by further experiments, an example of which is shown in table 2, which lists the detailed cytogenetic data of such an experiment and illustrates that D_2O induces chromosomal damage mainly of the chromatid type; that is, chromatid gaps and



Example of a D_2O-induced 'pulverized' metaphase.

breaks, without concomitant induction of chromatid interchanges, which are an otherwise rather common type of 'spontaneous' aberration in patients with Fanconi's anemia and Bloom syndrome.

It is clear from our experiments that short-term exposure to high levels of D_2O can induce chromosomal aberrations in human lymphocyte cultures. Despite the considerable variation that seems inherent to this system, of a total of 10 experiments in which cultures from Fanconi's anemia patients were compared to those from healthy individuals, the effect in a Fanconi's anemia patient was in no case less than that in a control culture tested simultaneously. We conclude therefore, that Fanconi's anemia lymphocytes have an increased sensitivity to the cytogenetic toxicity of D_2O.

Discussion. The mechanism of D_2O toxicity is poorly understood. Toxic effects may arise from various types of isotope effects at sites where hydrogen is exchanged for deuterium, along with D_2O solvent effects, presumably causing a slowdown of enzymatic reactions eventually resulting in a generalized derailment of cellular metabolism[2,12]. Mechanisms of this kind could also play a role in the generation of D_2O-induced cytogenetic effects described here. However, yet another type of mechanism might be envisaged, based on the observations that D_2O, compared to H_2O, as a solvent supports longer lifetimes of

Table 1. Cytogenetic effect of D_2O treatment in human lymphocyte cultures[a]

	Cells with chromosomal breakage[b]/cells scored		
	No treatment	H_2O-medium	D_2O-medium
Healthy individuals[d]			
Individual 1, experiment 1	5/100	5/100	6/100
experiment 2	5/100	7/100	13/100
experiment 3	0/100	–	38/100
Individual 2[c]	1/100	4/100	2/9
Individual 3	–	1/100	12/100
Mean percent cells with aberrations	2.8%	4.3%	17.4%
Mean number of breaks per aberrant cell	1.2	1.0	1.3
Bloom syndrome[d]			
Patient MaPa[c]	29/100	22/100	49/100
Patient AnPa[c]	30/100	25/100	40/100
Patient SuSc[c]	25/100	36/100	8/21
Patient StSc[c]	27/100	21/100	13/35
Mean percent cells with aberrations	27.8%	26.0%	43.0%
Mean number of breaks per aberrant cell	1.3	1.2	1.7
Fanconi's anemia[d]			
Patient JdW, experiment 1	13/100	36/100	39/100
experiment 2	18/100	21/100	30/50
experiment 3	13/100	25/100	35/50
Patient ST	5/100	6/100	14/37
Patient MM[c]	22/100	–	53/100
Patient MB[c]	9/100	17/100	54/100
Mean percent cells with aberrations	13.3%	21.0%	51.5%
Mean number of breaks per aberrant cell	1.1	1.3	2.1

[a] H_2O and D_2O media were supplemented with fetal calf serum components. [b] Chromatid gaps, chromatid breaks and isochromatid breaks only were considered (for explanation, see text). [c] For these cultures the period of time in H_2O or D_2O medium was 4 h; it was 5 h for all other cultures. [d] To obtain an impression of the statistical significance of the differences observed, the distribution-free Wilcoxon's signed rank test (one-sided) was used. For the pooled data, to test the hypothesis whether D_2O treatment has a cytogenetic effect in human lymphocyte cultures, the tail probability was < 0.005; the group with Bloom syndrome was too small for this test to be tested separately; for the healthy individuals and the Fanconi's anemia patients the tail probabilities for the difference between untreated and D_2O-treated were 0.05 and 0.025, respectively.

Table 2. Spectrum of D_2O-induced chromosomal aberrations

	Number of metaphases scored	Aberrations Chromatid type				Chromosome type				Number of metaphases with one or more aberrations
		Gaps	Breaks	Triradials	Quadriradials	Gaps	Breaks	Dicentrics	Rings	
Healthy control										
Untreated	100	6	3	0	0	1	0	0	0	7
H_2O-medium	100	10	3	0	0	0	0	0	0	13
D_2O-medium	100	45	34	0	0	0	2	0	0	44
Fanconi's anemia patient JdW										
Untreated	100	8	20	2	3	2	5	0	1	28
H_2O-medium	100	8	4	0	0	0	1	0	0	12
D_2O-medium	100	37	118	0	1	6	25	0	0	78

superoxide and singlet oxygen[13,14]. As these activated oxygen species are continuously generated during normal aerobic metabolism[15], it seems possible that the toxic action of D_2O on aerobically living cells may be partly based on the potentiation of oxygen toxicity. It is interesting to note that activated oxygen species have been implicated in the generation of 'spontaneous' chromosomal breakage in Fanconi's anemia[16] as well as in Bloom syndrome[17]. The increased sensitivity of Fanconi's anemia lymphocytes to the chromosome-breaking effect of D_2O, as indicated by our experiments, may thus suggest the existence of an oxygen-dependent mechanism for D_2O (geno)toxicity.

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Formation of the active antifertility metabolite of (S)-α-chlorohydrin in boar sperm

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Summary. The male antifertility agent (S)-α-chlorohydrin (**I**) is metabolized by boar sperm to (S)-3-chlorolactaldehyde (**II**) by an enzyme that is involved in the oxidation of glycerol to glyceraldehyde. The presence of glycerol decreases the activity of this enzyme towards (S)-α-chlorohydrin in vitro thereby preventing the formation of (S)-3-chlorolactaldehyde, an inhibitor of glyceraldehyde 3-phosphate dehydrogenase in boar sperm.

(R,S)-α-Chlorohydrin (3-chloropropan-1,2-diol, **I**) is an antifertility agent that inhibits glycolysis in mature sperm of the ram, guinea-pig, hamster, boar, rat, rhesus monkey and human[2,3]. When the syntheses of the separate isomers of α-chlorohydrin were achieved, this action was shown to be due solely to the (S)-isomer both in vivo[4] and in vitro[5], the site of action involving the inhibition of glyceraldehyde 3-phosphate dehydrogenase[3,5,6]. As the action of (S)-α-chlorohydrin in vitro was not immediate but was evident only after a period of incubation with mature sperm, it was suggested that a metabolite was the actual inhibitory compound[7]. Subsequently, this metabolite was detected in incubates of boar sperm[8] and identified as (S)-3-chlorolactaldehyde (**II**)[9] which is stereochemically identical to (R)-glyceraldehyde-3-phosphate (**III**), the substrate for glyceraldehyde 3-phosphate dehydrogenase[5]. We now present evidence that the formation of this inhibitory metabolite in boar sperm requires an enzyme that is involved in the oxidative metabolism of glycerol.

Washed boar sperm[10] were incubated in phosphate-buffered saline in Warburg flasks at 34 °C for 1 h with uniformly-labeled ^{14}C-substrates and the metabolically-derived $^{14}CO_2$ collected and assayed by standard procedures[11]. (S)-α-Chlorohydrin[12] (0.5 mM) inhibited the production of $^{14}CO_2$ by 90% when ^{14}C-fructose (1 mM) was the substrate but had no effect when ^{14}C-glycerol (2 mM) was the substrate. With ^{14}C-glycerol (2 mM) and (R,S)-3-chlorolactaldehyde (5 mM)[13], however, there was an 88% inhibition in $^{14}CO_2$ production. The oxidation of ^{14}C-glycerol-3-phosphate (2 mM) to $^{14}CO_2$ was similarly inhibited by (S)-α-chlorohydrin (0.5 mM) to the extent of 86% confirming that the presence of glycerol, but not of any of its metabolites on the pathway glycerol → glycerol-3-phosphate → dihydroxyacetone phosphate, was apparently preventing the oxidation of (S)-α-chlorohydrin to (S)-3-chlorolactaldehyde.

Two further experiments substantiated the involvement of glycerol metabolism in the oxidation of (S)-α-chlorohydrin. First, using ^{14}C-fructose (1 mM) as substrate, the inhibitory effect of (S)-α-chlorohydrin (0.5 mM) on $^{14}CO_2$ production was reduced from 90% to 12% when 1 mM glycerol was present and abolished in the presence of 10 mM glycerol. Second, when the sperm suspension was pre-incubated with (S)-α-chlorohydrin (0.5 mM) before ^{14}C-glycerol (2 mM) was added, there was inhibition of $^{14}CO_2$ production which increased with increasing time of pre-incubation.

These results can be accommodated by the scheme shown in the figure in which we propose that a pathway exists for the oxidation of glycerol to glyceraldehyde. In the absence of exogenous glycerol, (S)-α-chlorohydrin is converted by enzyme E_1 to (S)-3-chlorolactaldehyde which inhibits glyceraldehyde 3-phosphate dehydrogenase (E_2). In the presence of exogenous glycerol, the oxidative metabolite is not produced and there is no inhibition of E_2. However, when exogenous (R,S)-3-chlorolactaldehyde is added, E_2 is inhibited[15] thus preventing the oxidative metabolism of ^{14}C-fructose, -glycerol or -glycerol-3-phosphate to $^{14}CO_2$.

As to the identity of enzyme E_1, 2 candidates have been considered. Firstly, aldose reductases are known to interconvert a number of aldehydes and primary alcohols but