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Low molecular weight polyethylene glycol induces chromosome aberrations in Chinese hamster cells cultured *in vitro*

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The human population is widely exposed to polyethylene glycol (PEG) and its chemical derivatives, which are widely used as vehicles or co-solvents in many pharmaceutical and cosmetic preparations. However, PEG polymers of low molecular weight differ significantly from polymers of higher molecular weight in their physico-chemical properties, biological effects on cell permeability and their absorption and excretion, as well as their higher toxicity and possibly genotoxicity. In the present study we have analysed the induction of chromosome aberrations by the low molecular weight PEG polymers tetraethylene glycol (TEG), PEG 200 and PEG 400 in a Chinese hamster epithelial liver (CHEL) cell line, which retains sufficient metabolic capability to activate different promutagens and procarcinogens. The results indicate that in CHEL cells only TEG and PEG 200 are clastogenic. Parallel experiments performed in CHO cells in the presence and absence of rat liver S9 mix showed significant increases in chromosomal aberrations only in cultures treated with TEG in the presence of rat liver S9, indicating that low molecular weight polymers need to be activated to exert their genotoxic activity.

Introduction

Polyethylene glycols (PEGs) are a wide group of polymers of ethylene oxide very different from each other in their molecular weights and physico-chemical characteristics. PEGs with a molecular weight below ~600 are clear, viscous liquids, while at a molecular weight of ~1000 PEGs appear as white waxy solids. In general they are water soluble, stable, non-toxic compounds that do not hydrolyse or deteriorate on storage. Due to these characteristics, PEGs of different molecular weights (200–10 000) are widely used as vehicles or co-solvents in many pharmaceutical and cosmetic preparations. In animal studies PEGs have low oral and dermal toxicity. When administered orally the higher molecular weight PEGs appear to be less toxic than low molecular weight polymers because the latter are absorbed by the digestive tract, whereas larger polymers are absorbed more slowly or not at all.

The genotoxicity of PEGs is controversial. Negative results have been reported for PEG 6000 in a mouse lymphoma TK assay (Mitchell *et al*., 1997), for PEGs 300–400 in a sister chromatid exchange assay in CHO cells and in an unscheduled DNA synthesis assay (Anonymous, 1993) and for tetraethylene glycol (TEG) in the Ames test, in a mammalian mutagenicity test at the *HGPRT* locus and in a rat bone marrow cytogenetic assay (Union Carbide 1986, 1987a, 1988; Slesinski *et al*., 1989). However, positive results have been reported for TEG both with and without S9 mix in CHO cells (Union Carbide 1987b), but only at elevated dose levels that produced hyperosmotic conditions which have been shown to induce chromosomal damage (Galloway *et al*., 1987).

In the present work we aimed to assess the induction of chromosomal aberrations by TEG and other low molecular weight PEG polymers such as PEG 200 and PEG 400 in mammalian cells in culture. The PEGs selected have a variety of industrial applications. TEG is an industrial product used as a solvent/plasticizer and humectant, while PEG 200 and PEG 400 are included in cosmetics, in food as additives and in many pharmaceutical preparations as co-solvents or vehicles. For TEG the most probable human exposure would be occupational through dermal contact or, to a lesser extent, through inhalation, while for PEG 200 and PEG 400 the exposure would be through dermal contact, ingestion or injection (PEG 400).

The experiments were performed in an established Chinese hamster epithelial liver (CHEL) cell line which retains sufficient phase I and II metabolic capability to activate different classes of promutagens and procarcinogens (Turchi *et al*., 1987, 1992; De Salvia *et al*, 1988; Mosesso *et al*., 1994; Rueff *et al*., 1996; Biondi *et al*., 2000). Special attention has been devoted to keeping culture conditions within the physiological range due to the ability of PEGs to alter osmolality.

Materials and methods

Chemicals

Polyethylene glycol (CAS no. 25322-68-3) 200, PEG 400 and tetraethylene glycol (CAS no. 112-60-7) were purchased from Fluka AG (Switzerland). Since these chemicals are highly soluble in water, they were directly dissolved in culture medium after filter sterilization. 7,12-Dimethylbenz[*a*]anthracene (DMBA) (Sigma Chemical, St Louis, MO) and cyclophosphamide (CP) (Asta AG, Germany) were used as indirect positive control substances in the CHEL and CHO cells, respectively, in the presence of S9 mix. Mitomycin C (Kyowa, Hakko, Japan) was employed as a direct positive control. Mitomycin C and CP were dissolved in distilled water while DMBA was dissolved in dimethylsulphoxide (DMSO) (Fluka AG). The final concentration of solvent in the culture medium did not exceed 1% (v/v).

Cell lines and culture medium

CHEL cells were obtained as previously described (Turchi *et al*., 1987) and routinely cultured in William's medium supplemented with 10% fetal calf serum and antibiotics.

CHO cells were obtained from Prof. A.T.Natarajan (State University of Leiden, Leiden, The Netherlands) and cultured in Ham's F-10 medium supplemented with 15% newborn calf serum and antibiotics.

Both cell lines have a stable aneuploid karyotype with modal numbers of chromosomes of 24 and 21, respectively. Permanent stocks of cells are stored at –80°C and subcultures were prepared from stocks for experimental use. All incubations were at 37°C in 5% CO_2.

Selection of dose levels

Preliminary experiments were carried out using different dose levels of TEG, PEG 200 and PEG 400 with continuous treatment (16 h) in CHEL cells and of TEG and PEG 400 with short treatments (3 h treatment and 21 h recovery) in CHO cells. Cytotoxicity was assessed as a reduction in the mitotic

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indices and the confluence status of the cultures. Since the polymers assayed show low toxicity but rather strong osmotic activity, the criteria used in selecting adequate dose levels for cytogenetic analysis took into account the above-mentioned features of the chemicals and international standard recommendations that suggest avoiding increased osmolality of the culture medium. Accordingly, in the absence of particular restrictions due to toxicity, the upper limit selected did not exceed 10 mM, a limit designed to avoid artefactual increases in aberrations known to be caused by increased osmolality of the culture medium (Galloway *et al*., 1987, 1994; Seeberg *et al*., 1988). In some cases in CHO cells (e.g. TEG in the absence of S9 and PEG 400 in the absence and presence of S9), to show that the assay was exhaustive, the limit of 10 mM was exceeded because negative results were obtained in the absence of cytotoxicity.

Metabolic activation

Rat liver homogenate (S9 fraction) was obtained from young male rats following mixed induction with β-naphthoflavone and phenobarbital (Research Toxicology Centre, Pomezia, Italy). The S9 mix was prepared with the following composition and was added to the culture medium at a final concentration of 10%: 3 ml of S9 fraction; 1 ml of 40 mM NADP; 1 ml of 50 mM glucose 6-phosphate; 1 ml of 0.33 M KCl; 0.8 ml of 50 mM $MgCl_2$; 2 ml of 20 mM HEPES buffer; 1.2 ml of H_2O (total volume 10.0 ml).

Treatment procedure

CHEL (8×10^5) and CHO (3×10^5) cells were seeded in 25 cm^2 flasks 9–20 h before treatment under standard culture conditions. Then different concentrations of the test chemicals were added to each flask. In CHEL cells treatments with TEG, PEG 200 and PEG 400 were performed continuously for 16 or 24 h until harvesting. Untreated and solvent-treated cultures served as negative controls while DMBA served as a positive control.

In CHO cells short treatments (3 h) were carried out in the presence and absence of rat liver S9 mix with TEG and PEG 400 only. Short treatments were selected in this case because the presence of S9 does not allow long treatments. For CHO cells during treatment the concentration of serum was reduced to 2% to avoid a potential interaction with the test chemicals. This condition was also used with the negative control cultures. At the end of treatment cell monolayers were rinsed twice with fresh medium and reincubated under standard culture conditions for a further 21 h until fixation. The promutagen CP (6 μg/ml) and mitomycin C (0.07 μg/ml) served as positive controls in the presence and absence of S9, respectively.

During the last 2 h of culture colcemid was added at a final concentration of 0.05–0.1 μg/ml. Cells were then collected by trypsin/EDTA treatment, treated with hypotonic solution (tri-sodium citrate 1%), washed twice with fresh fixative, dropped onto glass slides and air dried. Slides were stained with 2% aqueous Giemsa and coded for cytogenetic analyses.

Cytogenetic analysis

Scoring of chromosome aberrations was performed in 100–200 well-spread metaphases per test point. Only metaphases with chromosome numbers that differed from the modal number by no more than two were considered. For statistical analyses of data the number of cells with aberrations excluding gaps was taken into account. The aberrations observed were allocated as chromatid or chromosome type. Both categories included breaks and exchanges and the percentage of aberrations was calculated. Results from repeated experiments are reported. Statistical analysis of data was performed with Fisher's exact test, accepting $P < 0.05$ as significant.

Results

The results for induction of chromosome aberrations by the three polymers of PEG (TEG, PEG 200 and PEG 400) in CHEL cells are presented in Table I. In Table I, for each dose level, the total number of cells scored, the frequency of chromatid and chromosome deletions and exchanges, the percentage of cells bearing aberrations excluding gaps and the relative mitotic index are reported. Of the three compounds assayed, TEG and PEG 200 induced marked and dose-related increases in aberration-bearing cells compared with the relevant solvent control values. Statistical significance was achieved for both TEG and PEG 200 at the three higher dose levels, although TEG appeared to be a stronger clastogenic agent than PEG 200 (29% aberrant cells at 5 mM TEG versus 13.3% at 8 mM PEG 200). The same trend was also observed at later sampling times (24 h), although the incidence of aberrant cells was slightly lower in absolute terms (data not shown). The aberrations found were mainly chromatid exchanges and breaks. In contrast, PEG 400 did not show any clastogenic activity up to the highest concentration employed (7 mM).

To determine whether PEG polymers require metabolic activation to exert their clastogenic effect, we also assessed TEG and PEG 400 in CHO cells in the presence and absence of rat liver S9 mix. The results obtained, presented in Table II, indicate that TEG induced marked and dose-related increases in aberration-bearing cells compared with the relevant control values both in the presence and absence of S9. Statistical significance was only achieved at the two higher dose levels employed, which were substantially different in the presence (1.1 and 1.7 mM) and absence of S9 (21.6 and 30.2 mM). It should be noted here that while in the presence of S9 mix TEG showed a very strong clastogenic response at relatively low dose levels, in its absence TEG induced chromosomal aberrations only at dose levels consistently exceeding the limit of 10 mM (21.6 and 30.2 mM), likely determined by 'secondary' effects generated by the non-physiological conditions. These findings corroborate the evidence that TEG requires metabolic activation to exert its clastogenic effects.

The large increase in mitotic index observed in the CHO experiment with TEG without S9 was apparently due to cell synchronization caused by, most probably, non-specific subtoxic effects of the test compound.

With PEG 400 significant increases in the frequency of aberrant cells were observed in the presence of S9, but only at dose levels exceeding the limit of 10 mM (25 and 35 mM). The positive control substances, CP and mitomycin C for CHO cells in the presence and absence of S9, respectively, and DMBA for CHEL cells, induced the expected frequencies of aberrations, indicating that the experimental system employed was functioning correctly.

Discussion

In this study we found CHEL cells to be a suitable metabolizing system for testing PEG polymers. In this system, in which the same cell acts as metabolic source and target for DNA damage, it is possible to use longer exposures at relatively lower dose levels, thus avoiding extraneous effects due to alterations in the physiological conditions of the culture medium (increased osmolality, pH, etc.).

Of the polymers examined, PEG 400 did not show any clastogenic activity in either CHEL or CHO cells in the presence and absence of S9 mix. It should be noted here that both the cytotoxicity and clastogenicity of the PEG polymers assayed progressively decreased with increasing molecular weight.

PEG 200 and PEG 400 are both mixtures of molecules of different sizes that differ in the number of oxyethylene units, with an average molecular weight of 200 and 400, respectively. Therefore, a likely conclusion of our results is that the toxic and clastogenic effects of PEG 200 are associated with a content of molecules with fewer oxyethylene units, such as TEG. PEG polymers below a given molecular weight should be able to enter cells and exert their genotoxic effect. This is related to the reduction in clastogenicity of PEG 200 observed in CHEL cells, compared with TEG, which could be associated with the content of TEG in PEG 200 (~31% TEG). Similarly, the considerable reduction in the content of TEG in PEG 400 (~0.7% TEG) and the relative increase in the length of its

Table I. Frequencies of chromosomal aberrations induced by polyethylene glycol polymers in CHEL cells

Dose (mM)	*n*	Aberrations per 100 cells		Aberrant cells (%)	Relative MI (%)
		Chromatid	Chromosome		
0	200	2.0	0.5	2.5	100
Tetraethylene glycol					
1.8	200	3.3	1.3	4.0	86
2.5	200	11.0	4.0	12.5[a]	73
3.5	100	10.0	3.0	18.0[a]	71
5.0	100	43.0	2.0	29.0[a]	71
7.0					44
Polyethylene glycol 200					
2.0	150	3.3	2.0	4.7	92
3.0	250	5.5	4.5	8.0[b]	83
4.5	150	6.7	2.7	8.0[a]	84
6.0	150	9.3	4.2	11.3[a]	76
8.0	150	13.3	4.7	13.3[a]	67
Polyethylene glycol 400					
7.0[c]	100	3.0	0	3.0	103
DMBA (3 μg/ml)	100	19.0	6.5	19.0[a]	42
DMSO (0.1%)	200	3.0	1.0	4.0	102

Continuous treatment for 16 h. *n*, total number of metaphases scored; aberrations, breaks + exchanges; aberrant cells (%), percentage of cells bearing aberrations (excluding gaps); relative MI, mitotic index relative to untreated control.
[a]Statistically significant at $P < 0.001$ (Fisher's exact test).
[b]Statistically significant at $P < 0.01$ (Fisher's exact test).
[c]Only the higher dose level assayed is reported.

Table II. Frequencies of chromosomal aberrations induced in CHO cells treated with polyethylene glycol polymers in the presence and absence of rat S9 mix

Treatment	S9	Dose (mM)	*n*	Aberrant cells (%)	Relative MI (%)
Tetraethylene glycol					
	+	0	200	3.0	100
	+	0.6	200	4.5	90
	+	1.1	200	8.0[a]	50
	+	1.7	100	20.0[b]	57
	−	0	200	4.5	100
	−	13.7	200	7.0	165
	−	21.6	100	26.0[b]	127
	−	30.2	100	48.0[b]	171
Polyethylene glycol 400					
	+	0	200	4.5	100
	+	17.0	175	4.0	103
	+	25.0	200	10.5[a]	96
	+	35.0	200	9.5[a]	81
	−	0	200	4.0	100
	−[c]	35.0	200	4.0	110
Cyclophosphamide	+	(6.00 μg/ml)	75	80.0[b]	34
Mitomycin C	–	(0.07 μg/ml)	100	44.0[b]	74

n, total number of metaphases scored; aberrant cells (%), percentage of cells bearing aberrations (excluding gaps); relative MI, mitotic index relative to untreated control.
[a]Statistically significant at $P < 0.05$ (Fisher's exact test).
[b]Statistically significant at $P < 0.001$ (Fisher's exact test).
[c]Only the higher dose level assayed is reported.

homologues could explain the absence of clastogenic activity of PEG 400.

Biochemical studies have demonstrated that mammalian alcohol dehydrogenase (ADH) is able to initiate the oxidation of different homologues of PEG 300 (Herold *et al*., 1989). This feature is consistent with the presence of dihydroxy and hydroxy acid metabolites of PEG 300 in the serum and urine of burns patients who died from a fatal form of poisoning after treatment with a PEG 300-based antimicrobial cream (Bruns *et al*., 1982). Therefore, one or more of these metabolites activated by ADH could have a DNA-damaging effect that is responsible for the clastogenic effects of TEG and PEG 200, as confirmed by the fact that ADH is expressed in CHEL cells and is active in rat liver S9 fraction.

The human risk associated with the low molecular weight PEGs assessed in this study is mainly due to skin exposure. Oral use of PEG 200, although it can be used as an excipient for tablets, is very limited, while exposure to TEG as the pure chemical is limited to occupational exposure, mainly through dermal contact. For PEG 200 and PEG 400 some concern exists for the general population as these chemicals are present in pharmaceutical and cosmetic products for topical use. Risk

assessment of these compounds is not a simple task as little information regarding current concentrations employed and systemic absorption is available. However, it seems that absorption through healthy skin is not very effective, although it can occur through damaged skin. For some known formulations in which the ointment base is 50% PEG 300, which contains 9% TEG, it is possible that, due to permeability of the skin to small molecules, a sufficient quantity of TEG could be absorbed locally, thus creating a genotoxic risk. This risk increases when these products are used on damaged skin.

The genotoxicity results for low molecular weight PEGs are also a warning sign for several PEG derivatives of similar polymeric size. The importance of evaluating the safety of these compounds is critical because they are present in many cosmetics and their use is chronic and extensive. For most of these agents, insufficient data on genotoxicity, adsorption and metabolism are available. However, recent studies on the clinical effects and permeability of various emulsifiers in human skin have demonstrated that low molecular weight PEG stearates, such as PEG 2 stearate and PEG 9 stearate (at a concentration of 5% w/v), are able to damage the skin barrier (Bàràny *et al*., 2000). In contrast, higher molecular weight PEGs (PEG 40 stearate) do not show these features. Thus the possibility of skin permeability to some low molecular weight PEG derivatives is an issue to be taken into account in the risk assessment of these compounds.

On the basis of our results we can conclude that PEG polymers with a molecular weight of ~200 have a genotoxic effect after metabolic activation as evaluated by induction of chromosome aberrations in CHEL and CHO cells in the presence of S9. These findings suggest a potential mutagenic risk also for PEG derivatives of similar polymer size.

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