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IS PURIFIED POLY(ETHYLENE GLYCOL) ABLE TO INDUCE CELL FUSION?

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Preparations of poly(ethylene glycol) 6000 (PEG) from five different commercial sources have been purified, and their ability to fuse hen erythrocytes has been investigated. Quantitative assessments of cell fusion showed that before purification one of the preparations (PEG Wako), was able to induce limited fusion (5–6%) of erythrocytes with conditions (1 min incubation with 50% w/w PEG) under which the other four unpurified preparations of PEG were inactive. On purification, PEG (Wako) became inactive. By contrast, when erythrocytes were incubated with 45% w/w PEG for 15 min, extensive fusion (23–27%) occurred with all five unpurified preparations of PEG. Under these conditions, the fusogenic properties of four of the preparations of PEG were unaffected by purification; fusion induced by PEG (Wako) was, however, decreased on purification from 27% to 19%. It appears that polymeric poly(ethylene glycol) is itself able to fuse cells, but that some commercial preparations, e.g. PEG (Wako), have enhanced fusogenic properties resulting from the presence of contaminating substances. No relationship between the absorbance at 290 nm of PEG and its fusogenic properties was found in this study. The addition of small quantities of fusogenic lipid-soluble compounds to PEG was, however, observed to enhance cell fusion by up to 50%.

Introduction

It has been reported by Honda et al. [1] that the fusion of human erythrocytes induced by poly(ethylene glycol) 6000, (PEG 6000), is decreased or even completely prevented when the polymer is recrystallized from chloroform and diethyl ether, and/or dialyzed against distilled water. These workers have suggested that diethyl ether-soluble and water-soluble contaminants in commercial PEG promote cell fusion, and that the contaminants may be antioxidants in view of the well-known autoxidation of PEG [2]. They have further proposed that PEG itself merely induces cell aggregation [1] and hence, when its contaminants are removed, PEG does not induce cell fusion. The possibility that PEG may not be intrinsically fusogenic is of considerable practical importance and interest, since the polymer has been employed for a number of years as a laboratory tool to induce cell fusion, and it is currently widely used to fuse lymphocytes with myeloma cells in the preparation of monoclonal antibodies. Furthermore, interpretations of experiments on mechanism(s) by which PEG induces membrane fusion (as, for example, in Refs. 3 and 4) may need to be reconsidered if fusion is to be attributed to contaminant molecules rather than to PEG itself.

We have therefore investigated PEG 6000 from several different commercial sources, and we report here that the majority (four out of five) of the unpurified, commercial preparations of PEG stud-

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Abbreviation: PEG, poly(ethylene glycol).

ied were unable to induce erythrocytes to fuse when they were used as described in [1]. When more appropriate conditions for the fusion of erythrocytes were employed, purification did not decrease the ability of four of the five preparations of PEG investigated to fuse the cells. There appeared to be no relationship between the absorbance at 290 nm of PEG and its fusogenic properties, contrary to the suggestion made by Honda et al. [1], but the addition of small quantities of fusogenic lipids to PEG has been observed to enhance cell fusion by up to 50% and this finding may have some practical applications.

Materials and Methods

Preparations of poly(ethylene glycol) 6000 were obtained as follows: PEG (Wako), batch No. DPG 0755, from Wako Pure Chemical Industries Limited, Osaka-shi, Japan; PEG (BDH), batch No. 367827, from BDH Chemicals, Poole, Dorset, U.K.; PEG (Koch Light), batch No. 85083, from Koch Light Laboratories, Colnbrook, Bucks. U.K.; PEG (Sigma), batch No. 81F9009, from Sigma Chemical Company, Poole, Dorset, U.K.; PEG (Nakarai), batch No. M9G3702, from Nakarai Chemicals Limited, Japan. All experiments with PEG (Wako) were undertaken with batch No. DPG 0755 unless otherwise stated.

Cell fusion was induced by incubating 0.01 ml of fresh, washed, packed hen erythrocytes in 0.2 ml 45% w/w PEG at 37°C in modified Eagle's medium [5] containing 5 mM Ca^{2+} for 15 min, followed by dilution with 10 ml of minimum essential Eagle's buffer containing 5 mM Ca^{2+} at 37°C, followed by a further incubation for 45 min. The cells were pelleted, fixed in 1 ml 2% glutaraldehyde for 1 h, washed in distilled water, stained with freshly filtered Harris' haematoxylin for 30 min, washed in tap water, and resuspended in ammoniated water. After counting, the percentage fusion was expressed as (number of multinucleated cells $\times$ 100) $\times$ (total number of cells)$^{-1}$. Six random fields containing at least 100 cells were counted per sample.

Results

Purification of poly(ethylene glycol) and cell fusion

In most of our experiments, hen erythrocytes were used rather than the human erythrocytes of Ref. 1, because the nuclei of hen erythrocytes make the quantitiation of cell fusion easier and more accurate. We have, however, also studied human erythrocytes and similar findings to those described here were made.

In initial experiments, we employed a short incubation period with PEG (1 min with 50% w/w PEG 6000 at 37°C followed by 5 min with 4.5% PEG), as in Refs. 1 and 6, to induce cells fusion. (Other experimental conditions were as described in the Materials and Methods section.) Only PEG (Wako), which was used in most of the studies by Honda et al. [1] (S. Sasakawa: personal communication), induced cell fusion under these conditions. The percentage of cell fusion ($\pm$S.D.) for hen erythrocytes incubated with unpurified PEG 6000 (Wako), (determined in triplicate in three separate experiments), was 5.3% ($\pm$0.3), 5.4% ($\pm$0.4) and 6.2% ($\pm$1.4). These data are comparable with those of Honda et al. [1], who reported from 4.3% ($\pm$2.1) to 8.2% ($\pm$3.2) cell fusion for six experiments with human erythrocytes incubated with PEG 6000 from various unnamed manufacturers. After its purification by dialysis or by recrystallization from chloroform/ether as described in Ref. 1 or from ethanol, we found that PEG 6000 (Wako) was unable to fuse erythrocytes, again confirming the findings of Ref. 1. By contrast, PEG (Koch Light), PEG (BDH), PEG (Sigma), and PEG (Nakarai) gave no cell fusion either before or after purification when used as in Ref. 1. These observations indicate that contaminants are present in the sample of PEG 6000 (Wako) investigated, which induce erythrocytes to fuse when they are treated for only 1 min with 50% w/w PEG, and that after purification the PEG (Wako) resembles unpurified samples of PEG from other sources. It is nevertheless interesting to note that PEG (Wako) was not significantly more haemolytic than PEG (BDH). Thus the percentage haemolysis observed with 45% PEG (Wako) in 5 min at 37°C in a modification of Eagle's basal salt solution [5] containing 5 mM Ca^{2+} was 28.6% ($\pm$8.4) before, and 24.2% ($\pm$5.7) ($n = 3$) after recrystallization from chloroform/ether; with 45% PEG (BDH) haemolysis was 23.1% ($\pm$4.7) before purification and 22.1% ($\pm$1.1) afterwards (all values $\pm$ S.D.).

In subsequent experiments, hen erythrocytes

were incubated with PEG for a longer period (15 min with 45% w/w PEG 6000 before dilution, as described in the Materials and Methods section), i.e., conditions that we originally described as being effective for the fusion of erythrocytes [7]. A comparison of the fusogenic properties of five different samples of PEG 6000 used in this way, before and after several different purification procedures, is given in Table I. All five samples gave extensive cell fusion (19–29%) under these conditions. Cell fusion induced by unpurified PEG (Wako) was slightly greater than that induced by unpurified PEG from other sources, and it was decreased slightly after purification. The fusogenic properties of the other four samples were, however, unaltered by purification. These data are consistent with the polymer being intrinsically fusogenic, and with the possibility that contaminants are present in unpurified PEG 6000 (Wako) that increase its ability to fuse cells. As we have confirmed the finding of Honda et al. [1] that purified PEG 6000 (Wako) does not fuse cells when used as in Ref. 1, it is unlikely that we failed to remove contaminants that may have been active fusogens in the experiments of Table I (particularly as the samples from Koch Light and from BDH were recrystallized from chloroform/ether and then dialyzed).

Samples of PEG 6000 (Wako), batch No. EWL 4364, before and after purification, were kindly provided by Dr. S. Sasakawa to enable us to compare their behaviour in our hands with that reported in Ref. 1. Although not enough of this material was available for quantitative estimations of cell fusion to be made, we found that its fusogenic properties before and after purification were qualitatively closely similar to that described here for PEG (Wako), batch No. DPG 0755.

Ultraviolet absorption

Some of the samples of commercial PEG examined by Honda et al. [1] were reported to have an absorption band at 290 nm, but no extinction coefficients were given. In the light of their observations, Honda et al. [1] concluded that there appeared to be three contaminants in PEG causing cells to fuse: (1) the substance which is soluble in ether and has an ultraviolet absorption band; (2) the substance which is soluble in ether but has no ultraviolet absorption band; and/or (3) the substance which is soluble in water and has no ultraviolet absorption band. Further, they suggested that these may be assigned either to phenolic antioxidants like butylated hydroxyanisole or to

TABLE I

PERCENTAGE FUSION OF HEN ERYTHROCYTES TREATED WITH DIFFERENT PREPARATIONS OF PEG 6000

Cell fusion was induced and assayed as described in the Materials and Methods section. The values shown are the means ± S.D. of three independent experiments. In each of these experiments, the cells were incubated with PEG in duplicate. For the purification of PEG with ethanol, PEG (20 g) was dissolved in warm A.R. grade ethanol (600 ml), reprecipitated on cooling, filtered and dried under vacuum. For the purification of PEG with ethanol/diethyl ether the method of Törmälä and Tulikoura [12] was used.

Purification procedure		PEG (Koch light)	PEG (BDH)	PEG (Sigma)	PEG (Nakarai)	PEG (Wako)
Chloroform/ether	before purification	25.6 ± 3.4	23.4 ± 2.6	22.6 ± 0.5	23.8 ± 1.8	27.1 ± 0.9
	after purification	25.5 ± 2.7	22.2 ± 2.3	21.9 ± 0.6	22.6 ± 1.0	19.3 ± 0.9
Dialysis	before purification	24.5 ± 3.1	23.2 ± 2.7		23.5 ± 2.4	27.1 ± 0.9
	after purification	25.2 ± 2.1	24.1 ± 1.4		24.2 ± 0.5	20.4 ± 0.3
Ethanol	before purification	22.9 ± 1.4		23.1 ± 1.2	24.6 ± 3.0	28.8 ± 1.6
	after purification	21.9 ± 1.0		24.2 ± 3.1	21.9 ± 1.0	21.6 ± 0.9
Ethanol/ether	before purification			21.6 ± 0.4		
	after purification			22.6 ± 0.2		
Dialysis and chloroform/ether	before purification	20.7 ± 0.8	20.2 ± 1.3			
	after purification	22.0 ± 1.6	21.9 ± 0.8			

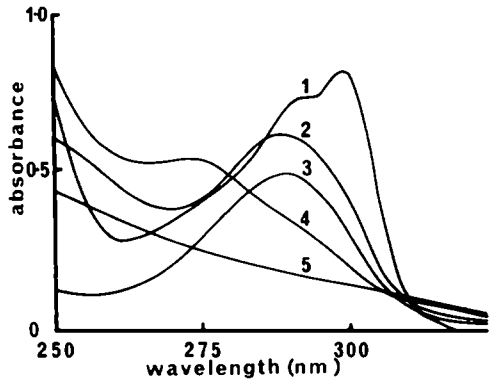

Fig. 1. Ultraviolet absorption spectra of PEG 6000 before purification. A 5% w/w solution in water of PEG (Wako) was used: all other samples were 20% w/w. Solutions were scanned between 250 and 325 nm in a Pye Unicam SP800 ultra-violet spectrophotometer (1 cm path-length). (1) PEG (Wako), (2) PEG (Sigma), (3) PEG (Nakarai), (4) PEG (Koch Light), (5) PEG (BDH).

the initiator or terminator of the polymerization of ethylene oxide. The ultraviolet absorption spectra of the unpurified preparations of PEG studied here are illustrated in Fig. 1. It is noteworthy that the absorbance at 295–305 nm of a 5% w/w solution of PEG 6000 (Wako), batch No. DPG 0755, is greater than that of 20% w/w solutions of any of the other four samples of PEG. This further supports the idea that PEG (Wako) contains fusogenic contaminants that increase the extent of fusion above that given intrinsically by PEG. However, there would appear to be no general relationship between the absorbance at 290 nm and the fusogenic properties of the unpurified polymers. PEG 6000 (Sigma) and PEG 6000 (Nakarai) absorbed quite strongly at this wavelength, while PEG 6000 (BDH) did not, despite the fusogenic properties of these three preparations being virtually indistinguishable (Table I). Furthermore, although PEG (Sigma) and PEG (Nakarai) exhibited a decreased absorbance after purification (Fig. 2), the cell fusion then induced was unchanged (Table I). (Unpurified PEG (Wako), batch No. EWL 4363, had an ultraviolet absorption spectrum similar to that shown in Fig. 1 for unpurified PEG from Nakarai Chemicals Ltd.)

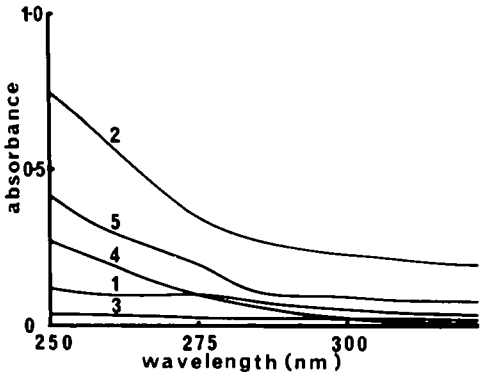

Fig. 2.Ultraviolet absorption spectra of PEG 6000 after purification, obtained as in Fig. 1 except that 20% w/w solutions were used throughout. (1) PEG (Wako) (reprecipitated from chloroform/diethyl ether), (2) PEG (Sigma) (reprecipitated from chloroform/diethyl ether), (3) PEG (Nakarai) (after dialysis), (4) PEG (Koch Light) (after dialysis), (5) PEG (BDH) (reprecipitated from chloroform/diethyl ether).

Enhancement of cell fusion

In Ref. 1 it was reported that addition to the purified polymer of the ether extract (obtained during recrystallization) partly restored fusogenic

TABLE II

EFFECTS OF ANTIOXIDANTS AND FUSOGENIC LIPIDS ON THE FUSION OF HEN ERYTHROCYTES INDUCED BY PURIFIED (DIALYZED) PEG 6000 (BDH)

Each compound tested was added to Eagle's minimal essential medium buffer or to the solution of PEG at a concentration of 50 μg/ml. Water-soluble antioxidants were dissolved in buffer or in PEG solution. Retinol was dispersed from ethanolic solution as described in Ref. 5, and other lipid-soluble compounds were dispersed by sonication using a probe ultrasonicator for 1–2 min. All compounds alone gave 0% fusion.

Addition	Percentage fusion
PEG alone	22.0 ± 0.8
+ *n*-propyl gallate	21.7 ± 0.9
+ methyl hydroquinone	22.5 ± 0.4
+ ethoxyquin	22.3 ± 0.6
+ butylated hydroxyanisole	27.7 ± 1.3
+ butylated hydroxytoluene	34.3 ± 1.9
+ glycerol monooleate	32.2 ± 0.9
+ α-tocopherol	34.7 ± 2.4
PEG alone [a]	13.2 ± 0.6
+ retinol [a]	19.6 ± 0.6

[a] Because of the lytic effect of retinol, cells were incubated for 30 min after dilution instead of 45 min (both with PEG alone and with retinol/PEG). This resulted in a generally lower incidence of fusion.

properties to the PEG. We similarly found that the addition of a concentrate from the ethanol extract of PEG 6000 (Wako) to the purified polymer restored weak fusogenic properties, viz. 3.9% ± 2.4 (S.D.) when assayed as in Ref. 1; the concentrate also slightly increased cell fusion when used as in the experiments of Table I. We have also confirmed the report in Ref. 1 that the antioxidants, α-tocopherol and butylated hydroxyanisole, enhance cells fusion by PEG 6000. Added α-tocopherol (50 μg/ml of PEG solution) was readily removed by extraction with chloroform/ether, but not by dialysis, and the extracted polymer then had its original fusogenic activity. In addition, we have confirmed that butylated hydroxytoluene and retinol (personal communication from R.A. Schlegel and J.W. Wojcieszyn) enhance cell fusion (Table II); we have also observed that glycerol monooleate enhances PEG-induced cell fusion (Table II).

Discussion

We have previously reported that glycerol monooleate, retinol and α-tocopherol are themselves able to fuse hen erythrocytes [5], although only at much higher concentrations than used in the present work. It thus appears that the fusogenic properties of the lipid-soluble fusogens studied here (glycerol monooleate, retinol, α-tocopherol) and of PEG are synergistic. Conceivable, this may be of practical value in some applications of cell fusion since, as in shown in Table II for α-tocopherol, the percentage cell fusion induced by PEG can be increased by 50% on the addition of a small quantity of this lipid fusogen. It seems, however, that the enhancements of cell fusion by α-tocopherol, butylated hydroxyanisole and butylated hydroxytoluene are not related to their antioxidant properties, since the water-soluble antioxidants *n*-propyl gallate (cf. Ref. 1), methyl hydroquinone, and ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline), when tested at comparable concentrations, did not enhance cell fusion (Table II). Hence although methylhydroquinone, which absorbs strongly at 290 nm, has been observed in Carbowax (high molecular weight PEG) [8], this antioxidant is unlikely to enhance cell fusion, even though it may be responsible for a high ultraviolet absorption in some samples of PEG.

It is important to note also in this connection that glycerol monooleate and retinol enhanced PEG-induced cell fusion. These two compounds are susceptible to oxidation because of their unsaturation, and they are not antioxidants. Indeed, retinol is very readily autoxidized by molecular oxygen, particularly when it is dispersed in aqueous solution [9].

The primary conclusion drawn from our investigations is that PEG is itself able to fuse cells. On the basis of work undertaken previously in this laboratory, it was suggested that the ability of PEG to bind water and thus to decrease the free water content of its aqueous solutions is important in causing cell fusion [10]. Both in that work, and in our more recent studies on changes in the permeability properties of liposomes that accompany their fusion by PEG [4], two preparations of PEG (from BDH and from Koch Light) were used, both of which have been shown here to be unaffected by purification in their fusogenic behaviour towards erythrocytes, unlike the behaviour of PEG (Wako) when used as described in Ref. 1. In view of our present findings, caution should perhaps be exercised in attributing observations made with PEG, as in Ref. 11, to contaminating molecules. Nevertheless, depending on the specific experimental conditions and preparation of PEG employed, the extent of cell fusion obtained in some applications of the polymer may depend to a greater or lesser degree on the enhancement of fusion by contaminant molecules. Effects of this kind may perhaps be partially responsible for the variability found in the behaviour of PEG in different laboratories in the production of hybridomas.

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