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SURFACTANT-INDUCED CELL TOXICITY AND CELL LYSIS

A STUDY USING B16 MELANOMA CELLS

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Abstract—The effects of a variety of detergents (non-ionic, ionic and bile derivatives) on B16 melanoma cells have been examined. Two main effects can be clearly differentiated: loss of cell viability and cell lysis. Under our conditions, cell–surfactant interaction is highly dependent on the nature of the amphiphile (more specifically, on its critical micellar concentration). Loss of cell viability occurs at surfactant concentrations below the critical micellar concentration, i.e. the incorporation of detergent monomers into the cell membranes is enough to impair their barrier function, so that Trypan Blue is no longer actively secreted outside the cell. On the other hand, cell lysis only occurs at or near the critical micellar concentration of the detergent, i.e. when the bilayer–micelle transition may take place. Comparative studies using B16 cells and phospholipid vesicles indicate that the amount of detergent required to induce cell lysis is the same that produces disruption of the lipid bilayer. Thus, our results suggest that membranes are the primary target for the toxicologic effects of surfactants on cells. Moreover, they provide a rationale for the interpretation of other studies in this field: previous results from different laboratories are shown to fit very well our data.

Surface-active compounds may be toxic for plants and animals. Increasing levels of these substances in waste water and other environments in the biosphere is a cause of concern. On the other hand, surfactant toxicity may be profitably exploited, e.g. in the killing of microbial or tumor cells. The toxic effects of detergents have been the object of many *in vivo* studies (see Refs 1–3 for review); however, studies at the cellular level are more scarce. Previous work from this laboratory [4–8] has dealt with the effect of detergents on isolated membranes or membrane components; relevant results from these studies include the observation that detergent monomers may bind the membranes at sublytic concentrations, thereby changing the bilayer physical properties, including permeability, and the complex effects of surfactants on the activity of intrinsic membrane properties. The present paper attempts to link those molecular studies with the effects at the cellular level; this should contribute to provide a rationale for the *in vivo* studies.

A variety of detergents has been selected, representative of the main groups of soluble amphiphiles [9]. B16 melanoma cells have been chosen as surfactant targets, because of (i) convenient culture conditions, (ii) known membrane properties [10], and (iii) possibility of using high- and low-metastatic variants [11]. Surfactant effects have been explored at two levels: permanent membrane damage, detected as the inability to pump out Trypan Blue ("loss of cell viability"), and cell lysis, measured by the presence of proteins in cell-free filtrates or by a decrease in cell suspension turbidity. Our results demonstrate that loss of cell viability occurs at surfactant concentrations clearly below those producing cell lysis, and suggest that membranes are the main targets for the toxicological effects of surfactants on cells.

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MATERIALS AND METHODS

CHAPS was purchased from Boehringer-Mannheim (Mannheim, F.R.G.); all other detergents were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All were used without further purification. A molecular weight of 635 was assumed for Triton X-100 in all the calculations. Egg-yolk phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were supplied by Lipid Products (South Nutfield, U.K.). Large unilamellar liposomes (ca 100 nm in diameter) containing PC:PE:cholesterol (2:1:1 mol ratio) were prepared by the extrusion method of Hope *et al.* [12]. Trapped volume measurements, according to the above authors, indicate that our liposomes contain about 1.5 lamellae/vesicle.

The B16-F1 and B16-F10 cell lines from C57-B1/6 mouse melanoma were a gift from Dr M. F. Poupon (Laboratoire des Métastases, I.R.S.C., C.N.R.S., Villejuif, France). Cells were grown in disposable plastic Falcon flasks (75 cm^2, 250 mL), on a defined RPMI 1640 medium [13], supplemented with 10% foetal bovine serum, 10^6 I.U./L penicillin and 2.5 mg/L streptomycin. Final pH was adjusted to 7.4. Culture flasks were kept under 5% CO_2, relative humidity near saturation and at 37°.

Cells were transferred from monolayers into suspensions by treatment with 0.01% (w/v) trypsin,

2 mM EDTA in phosphate-buffered saline (PBS medium) [14] at pH 7.0. After 2 min, the trypsin solution was removed, and the cells became detached slightly afterwards. They were then resuspended in fresh culture medium and harvested by centrifugation at 1000 *g*, 5 min, at 4°.

For detergent treatments, suspensions containing 10^6 cells/mL in PBS medium were mixed with equal volumes of the surfactant solutions. After incubation for the appropriate length of time, cell viability or other parameters were immediately determined. Detergent incubations lasted between 2 and 30 min. A 2-min treatment was arbitrarily chosen as representative of a short exposure to the surfactant; preliminary experiments had shown that, in most if not all cases, equilibrium had been reached after 30 min.

Total cell counting was performed using conventional Bürker chambers 0.01 mm/0.0025 mm^2/0.04 mm^2. Cell viability was assessed by the Trypan Blue method. Suspensions containing 5×10^5 cells/ml in PBS medium were mixed with equal volumes of 0.01% Trypan Blue in the same buffer. The proportion of non-stained (viable) over total cell number was then estimated using a Bürker chamber.

Cell lysis was assessed by the presence of proteins in cell-free preparations. Cells were removed from the surfactant-treated suspensions by filtration through GSWP 02500 Millipore filters, 0.25 μm pore diameter. This method has been previously applied to test the solubilization of phospholipid vesicles [15]. Proteins were determined in the filtrates by a modification [16] of the Lowry method. Alternatively, cell lysis was measured as a decrease in cell suspension turbidity; the latter was measured as absorbance at 450 nm in a Uvikon 860 Kontron spectrophotometer against a blank of pure buffer. Liposome solubilization was equally measured as a decrease in suspension turbidity [7, 15].

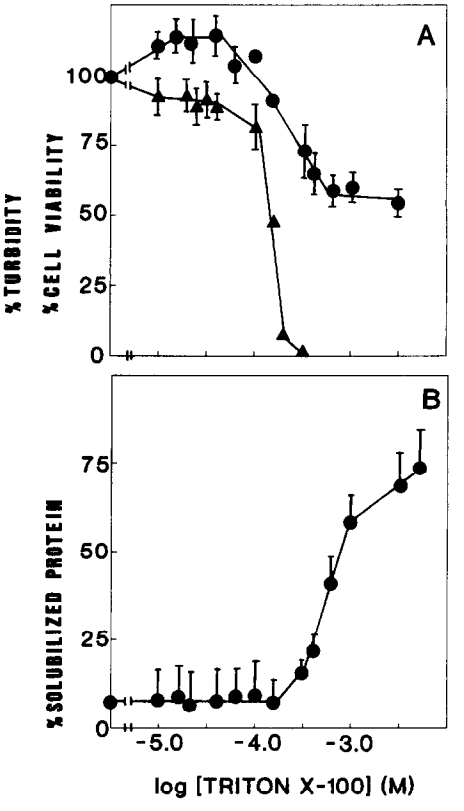

Fig. 1. The effects of Triton X-100 on B16-F10 melanoma cells. (A) Percentage variation of (▲) cell viability, and (●) cell suspension turbidity, as a function of surfactant concentration. (B) Percentage of solubilization of cell protein as a function of surfactant concentration. Detergent treatment took place for 2 min. Data points correspond to average values ± SE (N = 4). In each case, 100% is the value in the absence of detergent.

RESULTS

The effects of Triton X-100 on B16-F10 cells may constitute a representative example of cell–surfactant interaction. Figure 1 shows the variation in cell viability, cell suspension turbidity and cell protein solubilization, after exposure to various Triton X-100 concentrations for 2 min. Cell viability (Fig. 1A) changes only slightly with detergent concentrations up to 10^{-4} M, then decreases abruptly, reaching a value of 50% for 1.3×10^{-4} M surfactant. This kind of behaviour has been found with all other detergents and cells tested (see below). Therefore, we have defined the "maximum tolerable concentration" of detergent as the highest concentration of amphiphile not producing a significant decrease in cell viability. This parameter is obviously dependent on cell concentration, and must be considered a semi-quantitative approximation rather than a fixed value.

Cell suspension turbidity also decreases in a rather abrupt way, although at surfactant concentrations higher than required to decrease cell viability (Fig. 1A). A slight increase in turbidity is observed at low detergent values with virtually all the surfactants under study, and is probably due to partial cell aggregation and/or fusion [7]. Cell protein solubilization starts at the same point as the decrease in turbidity, yet it appears to be more gradual. The half-points of turbidity change and protein solubilization occur at 2.5×10^{-4} M and 6.3×10^{-4} M Triton X-100 respectively. This may suggest that variations in turbidity reflect the disruption of the cell membraneous structures rather than the complete solubilization of cell components. (Solubilization, in this context, refers to the operational definition that considers as solubilized all non-sedimentable material after centrifugation at 100,000 *g*, for 1 hr [5]).

The kinetics of detergent effects has also been considered, by measuring cell viability at various times, for up to 30 min. Preliminary experiments had shown little or no changes that could be attributed to the surfactant after this period. The detergents under study could be classified into two groups, from the kinetic point of view: fast-acting, i.e. those whose action is completed in 2 min, and slow-acting, that take a longer time to reach equilibration. Figure 2 presents representative examples of the fast and slow groups, respectively CHAPS and sodium cholate.

The various detergents are compared, from the different points of view mentioned above, in Table 1. A number of regularities may be observed: (i) all

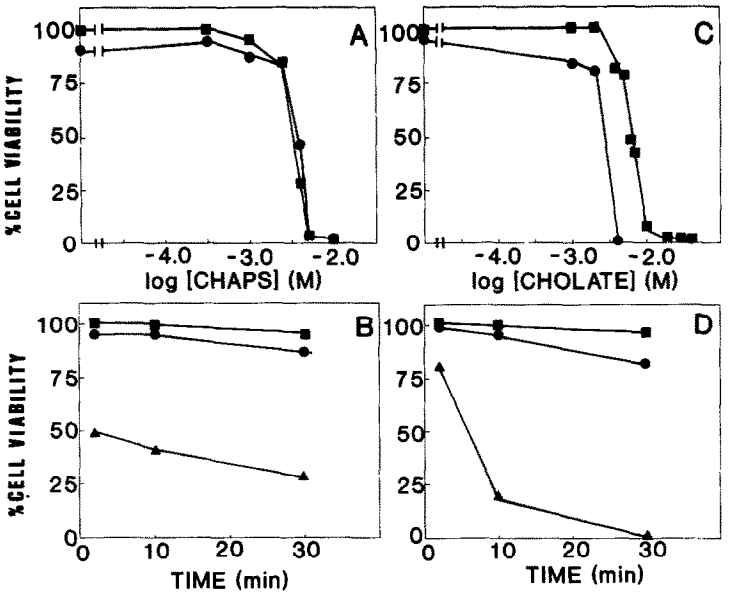

Fig. 2. The kinetics of detergent action on the viability of B16-F10 melanoma cells. (A) Percentage variation as a function of CHAPS concentration, after (■) 2 min and (●) 30 min treatment. (B) Percentage variation as a function of time, for different CHAPS concentrations: (■) control; (●) 10^{-3} M; (▲) 4×10^{-3} M. (C) Percentage variation as a function of Na cholate concentration, after (■) 2 min and (●) 30 min treatment. (D) Percentage variation as a function of time, for different Na cholate concentrations: (■) control; (●) 2×10^{-3} M; (▲) 4×10^{-3} M. Average values of two independent experiments.

Table 1. The effect of soluble amphiphiles on B16-F10 melanoma cells

	t = 2 min				t = 30 min		
	cmc*	mtc†	50% viability‡	50% turbidity‡	mtc†	50% viability‡	50% turbidity‡
Triton X-100	2.4×10^{-4}	1.0×10^{-4}	1.3×10^{-4}	2.5×10^{-4}	2.0×10^{-5}	4.0×10^{-5}	1.0×10^{-3}
Reduced Triton X-100	5.6×10^{-4}	1.0×10^{-4}	1.3×10^{-4}	ND§	2.0×10^{-5}	6.0×10^{-5}	ND§
Octylglucoside	2.5×10^{-2}	7.0×10^{-3}	1.0×10^{-2}	1.5×10^{-2}	$<6.0 \times 10^{-3}$	6.0×10^{-3}	1.2×10^{-2}
CHAPS	9.1×10^{-3}	2.5×10^{-3}	4.0×10^{-3}	1.0×10^{-2}	2.5×10^{-3}	4.0×10^{-3}	1.0×10^{-2}
Na cholate	1.0×10^{-2}	4.0×10^{-3}	7.0×10^{-3}	9.1×10^{-3}	2.0×10^{-3}	2.3×10^{-3}	7.0×10^{-3}
Na dodecylsulphate	8.3×10^{-3}	1.2×10^{-4}	2.5×10^{-4}	3.5×10^{-4}	1.2×10^{-4}	1.6×10^{-4}	2.6×10^{-4}
CTAB	9.2×10^{-4}	1.0×10^{-5}	1.8×10^{-5}	—	1.0×10^{-5}	1.5×10^{-5}	—

A summary of experimental data, after detergent treatments for 2 or 30 min. All concentrations are expressed in molarities.

* Critical micellar concentration. Data from [20], except for reduced Triton X-100 (S. Paredes, personal communication), CHAPS [21] and sodium cholate [22].

† "Maximum tolerable concentration". See text for definition.

‡ Molar concentration of detergent producing 50% decrease in cell viability, or in cell suspension turbidity. Data obtained by interpolation from plots of the kind shown in Fig. 1. Estimated experimental errors are less than 10% the values given in the Table.

§ ND: not determined.

surfactants have their "maximum tolerable concentrations" below their critical micellar concentrations, (ii) the decrease in cell viability always occurs at concentrations lower than those decreasing cell suspension turbidity, (iii) the toxic and lytic detergent concentrations depend on the nature of the amphiphilic molecule, (iv) some surfactants (CHAPS, Na dodecylsulphate, CTAB) appear to equilibrate with the cell system faster than others, and (v) ionic detergents (Na dodecylsulphate, CTAB) impair cell viability at concentrations, relative to their cmc, much lower than other detergents (about 100-fold). Two additional points deserve our attention. Reduced Triton X-100 is very similar to the parent compound, except that the benzene ring has been hydrogenated to a cyclohexane [17]. It may have a number of important applications, particularly because it does not interfere with protein

Table 2. A comparative study of the effects of Triton X-100 and octylglucoside on B16-F1 cells, B16-F10 cells, and large unilamellar vesicles composed of phosphatidylcholine, phosphatidylethanolamine and cholesterol (2:1:1 mol ratio)

	50% viability	50% turbidity	50% solubilization
		Triton X-100 (M)	
B16-F1	2.0×10^{-4}	3.6×10^{-4}	5.0×10^{-4}
B16-F10	1.3×10^{-4}	2.5×10^{-4}	6.3×10^{-4}
LUV	—	4.6×10^{-4}	—
		Octylglucoside (M)	
B16-F1	1.2×10^{-2}	1.7×10^{-2}	1.8×10^{-2}
B16-F10	1.0×10^{-2}	1.5×10^{-2}	1.7×10^{-2}
LUV	—	1.6×10^{-2}	—

Values obtained by interpolation from plots of the kind shown in Fig. 1. Estimated experimental errors are less than 10% of the values given in the table.

fluorescence or infrared absorption [18]. It is interesting to observe that, at least from the point of view of the interaction with B16 cells, both regular and reduced Triton X-100 are virtually indistinguishable. In addition, CTAB is peculiar in that, at least at concentrations up to 10^{-2} M, it does not cause any significant decrease in turbidity, probably because membrane lysis is being compensated by some kind of aggregation phenomenon.

The effect of some detergents was also compared on two kinds of B16 cells, namely B16-F1 and B16-F10, respectively low- and highly-metastatic [11]. This particular experiment was designed because of previous reports [10] on different membrane properties of both variants, and also because of the potential interest of selectively destroying the more metastatic cells. Our results are summarized in Table 2. Although B16-F10 cells appear to be slightly more sensitive to both Triton X-100 and octylglucoside, it is doubtful that the observed differences can be of practical use. The lower cholesterol/phospholipid ratio of B16-F10 cells may explain their higher sensitivity towards surfactants [10, 19]. The comparison with pure lipid vesicles (large unilamellar vesicles, LUV) is interesting. These vesicles, containing PC, PE and cholesterol at a 2:1:1 mol ratio, were suspended at a final concentration equivalent in phospholipid to 10^6 cells/mL, i.e. the cell concentration used in the above experiments. The data in Table 2 indicate clearly that the amount of detergent that produces a 50% decrease in turbidity is exactly the same for pure lipid vesicles and for whole cells. This observation supports our previous suggestion that changes in turbidity reflect primarily the disruption of membrane lipid bilayers.

DISCUSSION

The above results point clearly towards the existence of two separate effects of detergents on cells: loss of solute-barrier capacity of the membrane and lysis of cell integrity. Both are, in fact, membrane effects, and membranes can be considered as the primary targets for detergent toxicity in cells. Increases in "permeability", "drug absorption", etc. have been repeatedly pointed out as toxicologic effects of surfactants [3, 23–26].

Cell viability is assessed as the capacity to actively transport Trypan Blue outside the cell. The dye being a non-polar, negatively-charged molecule, diffuses out by means of an electrophoretic process, driven by the cell resting potential. As soon as the membrane does no longer constitute a diffusion barrier for the monovalent ions that are primarily responsible for the resting potential, the latter vanishes, and Trypan Blue equilibrates freely inside and outside the cell. It could be argued that the primary lesion occurs at the level of the sodium pump, or other membrane carriers also involved in the maintenance of the resting potential, but our previous studies on mitochondrial [27] and sarcoplasmic reticulum [5] membranes indicate that the solute-barrier properties are lost at low-detergent concentrations, that do not inhibit, or even enhance, the activity of membrane integral proteins.

In the course of a systematic study of detergent effects over 11 different cell lines, Tobler *et al.* [28] have found that the surfactant concentration required to produce a given effect depends upon the detergent structure rather than upon the cell type investigated. Similar observations are shown in our Table 2. However, to the authors' knowledge, previous workers have not pointed out the relationship, that is obvious from our Table 1, between toxic concentrations and critical micellar concentrations (cmc) of detergents. Concentrations producing a 50% decrease in cell viability are clearly cmc-linked, and always below that critical value. This means that incorporation of detergent monomers is enough to alter significantly the permeability barrier properties of the plasma membrane. Ionophoric properties of Triton X-100 at sub-lytic concentrations have been described in black-lipid films [29]. (The cmc values listed in Table 1 have been obtained in pure water, and they may change in the PBS medium in which cells are suspended, but not so much as to invalidate our previous conclusion.)

Other authors have used different criteria for cell viability. Their results are remarkably similar to ours. Ferguson and Prottey [30] studied the Na dodecylsulphate concentrations required to inhibit DNA synthesis and to produce cell "lysis" in guinea-pig fibroblasts. In their case, 2.2×10^{-4} M Na dodecylsulphate produces 50% cell "lysis" measured as

release of ^{51}Cr (compare with our results in Table 1).

Cell lysis may be investigated either by a decrease in cell suspension turbidity or by the presence of proteins in cell-free filtrates. Both methods give very similar results (Table 2), the change in turbidity occurring at slightly lower surfactant concentrations. Turbidity decreases at detergent concentrations near the cmc, i.e. when the surfactant is able to solubilize membrane components [7, 15]. It is significant that the amphiphile concentrations required to solubilize B16 cells coincide with those solubilizing liposomes composed of an equivalent amount of phospholipids (Table 2). This is in agreement with our previous studies on liposome solubilization by various kinds of detergents [8].

Previous studies on the effect of surfactants on tumour cells may also be interpreted in the light of our observations. Tarnowski *et al.* [32] find that 1.5×10^{-4} M Na dodecylsulphate reduces by 50% the capacity of mouse ascites tumour cells to proliferate.

The surfactant-induced increase in permeability of ascites tumour cells has also been noted [23]. On the other hand, the lack of correlation between *in vivo* antitumour activity and *in vitro* haemolytic properties of some surfactants [2, 3] in no way contradicts our conclusions, since the pharmacokinetics of the detergents, apart from being virtually unknown, must certainly be very complex.

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