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Interactions of surfactants with living cells. Induction of apoptosis by detergents containing a β-lactam moiety

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Summary. The toxicity of new surfactants containing a β-lactam ring has been established by studying their interaction with a hybridoma cell line. An hour of contact is sufficient to generate an apoptotic signal after two days of culture. Under the experimental conditions chosen for the experiments, surfactants have been divided into three categories: i) biocompatible and non-apogenic; ii) surfactants triggering an apoptotic signal without inducing cell necrosis; iii) surfactants triggering an apoptotic signal at low concentrations and destroying the cells by necrosis at higher concentrations. The necrosis inducing surfactants also had haemolytic properties. These properties were related to the values of the hydrophilic-lipophilic balance of the molecules.

Keywords: Amino acids – Surfactants – Toxicity – Apoptosis

Abbreviations: DMSO d-6 deuteriated dimethylsulphoxide, DNA deoxyribonucleic acid, HEPES hydroxyethyl-piperazin-ethylsulfonate, HLB hydrophilic-lipophilic balance, N necrotic cells, NVA non-viable apoptotic cells, PBS phosphate buffer solution, RPMI Roswell Park Memorial Institute, RNA ribonucleic acid, VA viable apoptotic cells, VNA viable non-apoptotic cells.

Introduction

Surfactants play a major role in numerous biological processes. Natural surfactants (phospholipids) have long been used as drug carriers (Lappalainen et al., 1994; Lawrence, 1994). More recently, cationic surfactants have been used to achieve lipofection, or to enable DNA, RNA and anti-sense oligonucleotide to ease their way into cells (Allen, 1994). Surfactants permitted also effective cell protection in bioreactors (Hua et al., 1993). They may be used as anti-bacterial agents for biological applications, provided their cell toxicity remains low.

The aim of the present study was to prepare a new series of biocompatible, anti-bacterial surfactants containing a β-lactam ring (see the formula in Fig. 1). Their ability to induce cell apoptosis or necrosis had to be determined to evaluate their toxicity. Their microbial activity has been published elsewhere (Gérardin-Charbonnier et al., 1999).

Apoptosis induction by artificial surfactants is poorly understood and to our knowledge only one study deals with the apoptotic activity of a synthetic surfactant (Borner et al., 1994). Low concentrations of toxic substances are generally assumed to be apogenic (i.e. inducing apoptosis) while higher concentrations have a necrotic effect (Kerr, 1993; Kroemer, 1997; Nicotera and Leist, 1997). The two phenomena are easily distinguishable on the basis of morphological studies after specific staining, as described by Mercille and Massie (1994).

In a first step we compared the apoptotic activity of the synthesized surfactants affecting hybridoma cells (HF2 $\times$ 653) in order to understand their toxicity better. Unlike many hybridomas, HF2 $\times$ 653 cells undergo spontaneous apoptosis only at the end of their culture. The toxicities found were compared to the haemolytic properties of the surfactants. We tried to find correlations between the hydrophilic-lipophilic balance (depending on the length of the hydrophobic chains) of the molecules and their toxicological properties.

Materials and methods

Materials

The surfactants were synthesized as indicated in the literature (Selve et al., 1991; Molina, 1995; Molina et al., 1995).

As the surfactants containing the glucosamine group were soluble in water, they have been used in PBS solutions. Because of the low solubility of the other surfactants, adequate volumes of a 5 g/L N,N-dimethylformamide solution were added to PBS to obtain the desired final concentrations. The chemical stability of the β-lactam ring and the ester bonds in aqueous solutions has been demonstrated by ^{13}C NMR.

Haemolytic activities

Haemolysis experiments were carried out by incubating the surfactant solution in phosphate buffer solution (PBS) for one hour at room temperature with human red blood

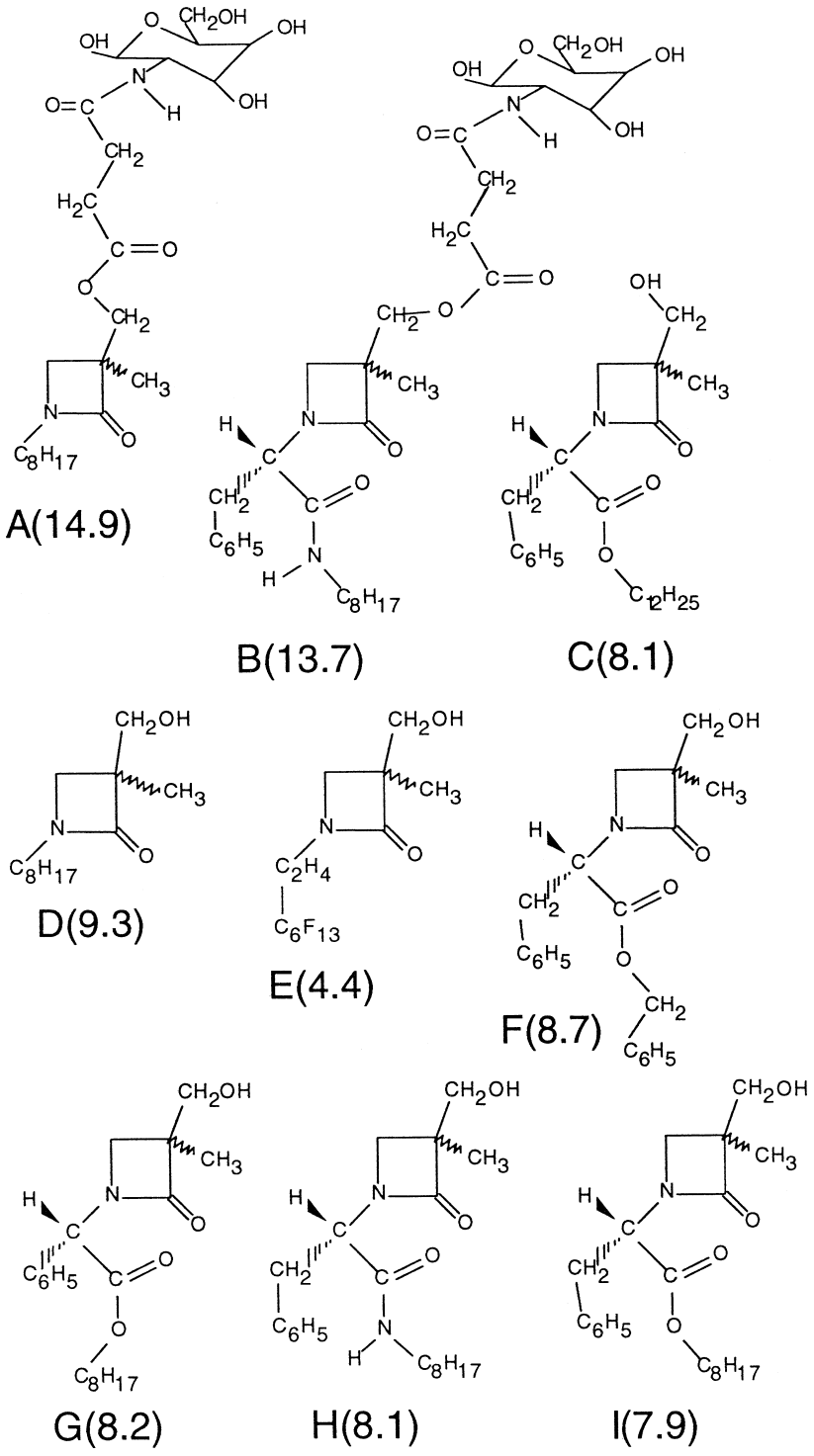

Fig. 1. Chemical structures of the different surfactants synthesized. All molecules were built around a β-lactam ring to which different groups were linked in order to modify the hydrophilic-lipophilic balance (HLB) of the compounds. Numbers in brackets beneath the chemical structures are the values of the HLB calculated as described in the literature (Griffin, 1955)

cells at a concentration of 10^9 per ml. The haemolysis percentages were obtained after centrifugation (100 g, 5 min) by measurement of the liberated haemoglobin (absorption at 414 nm, spectrophotometer UV-160 SHIMADZU, Kyoto, Japan). 100 and 0% of haemolysis were determined by incubation of red blood cells with water and with a control solution, respectively.

Toxicity towards mammalian cells

The mammalian cell toxicity was assessed as follows. An exponential culture of HF2 × 653 human/mouse hybridoma cells, (ECACC number 90012609, European Collection of Animal Cell Culture CAMR, Porton-Down, UK) was cultivated in the medium RPMI 1640 supplemented with 1 mM glutamine, 100 UI/ml penicillin G, 100 μg/ml streptomycin, 20 mM HEPES (hydroxyethyl-piperazin-ethylsulfonate) and 10% heat inactivated foetal calf serum. 10^6 cells were centrifuged and suspended in 5 ml of PBS. 5 ml of the surfactant solution at the appropriate concentration in PBS were added to 5 ml of the cell suspension. After one hour's incubation at 37°C, the cells were centrifuged and placed in 10 ml of culture medium. Cells were then incubated (37°C) for two days and then stained as described by Mercille and Massie (Mercille and Massie, 1994).

The cells were subsequently incubated for a short while with a mixture of acridine orange (a vital probe) and ethidium bromide (a non-vital probe). The nuclei of viable cells (both apoptotic and non-apoptotic) displayed a green fluorescence while normal or apoptotic nuclei in non-viable cells turned bright orange. Viable and necrotic nuclei showed variations of fluorescence intensities according to the presence of heterochromatin and euchromatin. The apoptotic nuclei were uniformly stained (high density chromatin). Cell counting and photography were performed by fluorescence microscopy (Axioskop ZEISS, Strasbourg, France) using an excitation bypass filter (450–490 nm), and an emission cut off filter (550 nm). The values presented were the average of at least three independent experiments.

Hydrophilic-lipophilic balance

Hydrophilic-lipophilic balance values (HLB) were calculated using the method described by Griffin (Griffin, 1955). For a given compound the HLB is calculated as the ratio of the molar weight of the hydrophilic moiety and the molar weight of the whole molecule, multiplied by 20.

Results

Only surfactants G, H and I exhibited haemolytic properties as soon as their concentration in the medium used for cell incubations exceeded 3.10^{-4} M. The results for the three surfactants are shown in Fig. 2.

The behaviour of HF2 × 653 hybridoma cells supplied by the European Collection of Animal Cell Culture has been studied by classical methods. The electrophoresis of DNA extracts did not show any fragments corresponding to 180 bp multiples of oligonucleotides. The apoptotic character of the cells appeared only at the end of the culture period.

The cells used to assess the toxicity of the surfactants were taken from suspensions in the exponential growth phase. The cells were centrifuged, washed, and suspended in PBS containing the surfactant under study at varying concentrations. They were incubated for one hour at 37°C. The cells

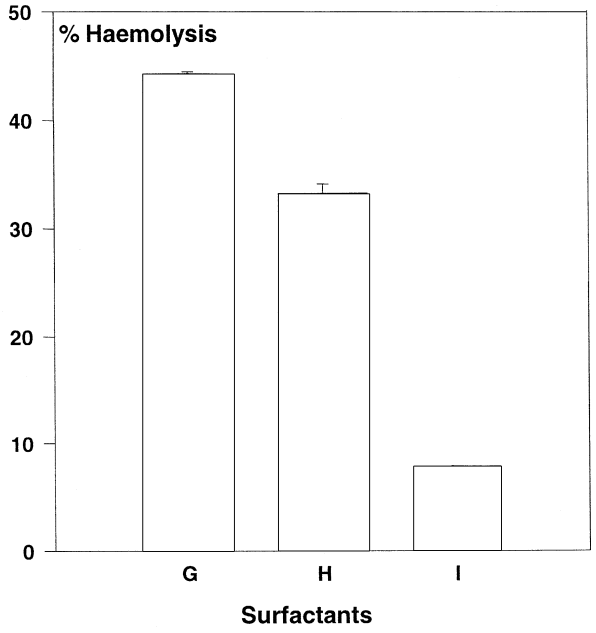

Fig. 2. Percentages of haemolysis induced by the three surfactants G, H, and I. The percentages were determined by measuring the haemoglobin liberated after red blood cell incubation in 3.10^{-4}M surfactant solutions for one hour (see section "Materials and methods")

Table 1. Criteria for the differentiation of cells stained with a mixture of acridine orange (AO) and ethidium bromide (EB)

Cell classification	Criteria	Nucleus	Cytoplasma	Designation
viable cells	AO enters the cells, EB does not, no apoptotic nor necrotic morphology	green	orange	$AO^+/EB^-/AP^-/N^-$
viable apoptotic cells	AO enters the cells, EB does not, apoptotic morphology and no necrotic features	green + chromatine condensation	orange	$AO^+/EB^-/AP^+/N^-$
non-viable apoptotic cells	AO and EB enter the cells, apoptotic morphology and no necrotic features	bright red + chromatine condensation	red	$AO^+/EB^+/AP^+/N^-$
necrotic cells	AO and EB enter the cells, no apoptotic morphology but necrotic features	dark red	red	$AO^+/EB^+/AP^-/N^+$

were then centrifuged and suspended in fresh complete medium, and cultured again for two days. Next they were stained with a mixture of acridine orange and ethidium bromide. The percentages of the different types of cells, classified as shown in Table 1, were evaluated as follows. Cells with characteristics $AO^+/EB^-/AP^-/N^-$ were designated as viable, those with

characteristics $AO^+/EB^-/AP^+/N^-$ and $AO^+/EB^+/AP^+/N^-$ as apoptotic, whereas those with characteristics $AO^+/EB^+/AP^-/N^+$ were designated as necrotic.

All the surfactants were used within the same molar concentration range. Their chemical stability in aqueous solution was verified by means of ^{13}C NMR. Therefore the observed toxicity was unlikely to be attributable to degradation products of the surfactants.

Surfactants A, B and C did not display any cell toxicity up to concentrations of 3.10^{-4}M, while surfactants D, E, and F brought about apoptosis above a certain concentration level. Surfactants G, H and I induced apoptosis or necrosis at all concentrations and may therefore be considered as cytotoxic. The cells were destroyed by necrosis as soon as the surfactant concentration reached approximately 10^{-4}M. All these results are summarized in Fig. 3.

Discussion

The cells were incubated in a medium containing the different surfactants and phosphate buffer (PBS) to avoid the formation of possible bonds between surfactants and proteins in the culture medium, (e.g. albumin) since such binding would have concealed the toxicity of the surfactants.

NMR spectroscopy has shown that the β-lactam ring and the ester bond of the surfactants were not hydrolysed, neither after 6 hours in aqueous solution (60% DMSO-d6, 40% H_2O) at 70°C nor after 48 hours at ambient temperature. Thus the observed toxicity is unlikely to be attributable to degradation products of the surfactants.

Classification of surfactants

Figure 3 shows that surfactants can be divided into three classes within the concentration range studied: Surfactants A, B and C have a very low cell toxicity level, surfactants D, E, and F induce an apoptotic signal in the cells (this phenomenon is concentration dependent), and surfactants G, H and I induce an apoptotic signal and cause cell necrosis as soon as the concentration attains a value of 10^{-4}M.

The link between toxicity and concentration (a compound may be apogenic at low concentrations and behave as a necrosing agent at higher concentrations) has been reported in previous literature (Kerr, 1993; Kroemer, 1997; Nicotera and Leist, 1997).

Haemolytic effects

Among all the surfactants studied, only the most cytotoxic compounds G, H and I have also haemolytic properties. The haemolytic and necrotic properties of the surfactants derive from the same mechanism, resulting in cell mem-

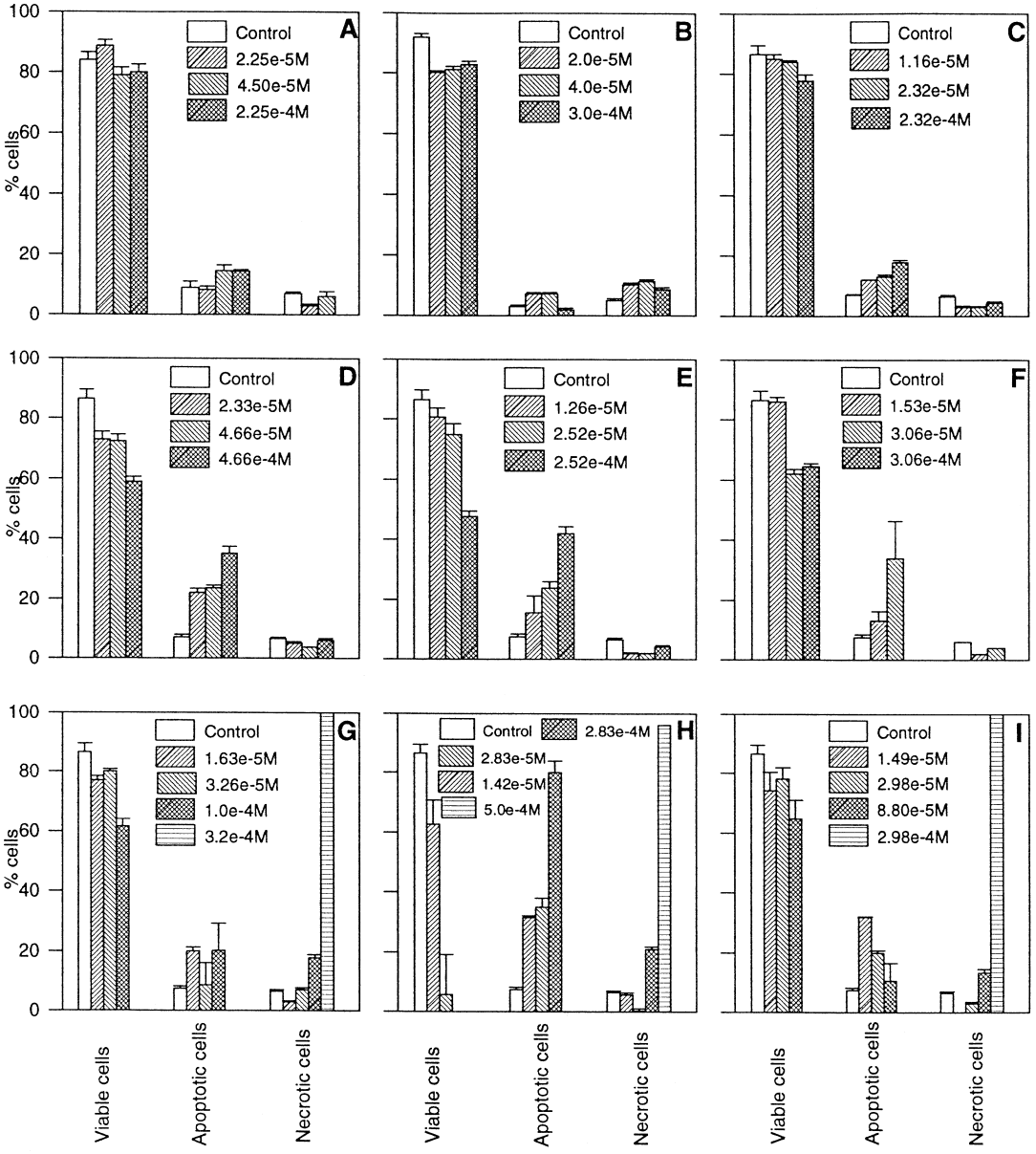

Fig. 3. Toxicity of surfactants. The percentages of the different cells were calculated by the method of Mercille and Massie (1994). Cells were incubated at various concentrations of surfactant and cultivated for two days before examination (see "Materials and methods"). The differentiation is based on the criteria listed in Table 1

brane destruction. This connection has not often been investigated in the recent past. I may be ascribed to the disrupted liquid crystal structure which gives rise to partial solubilisation of the cell membrane due to mixed micelle formation (Helenius and Simons, 1975; Lash, 1995).

Surfactant-membrane interactions

The haemolytic (and necrotic) properties of the surfactants were explained by the incorporation of the surfactants into the membrane. Since red blood cells do not have a metabolic activity which would permit programmed cell death leading to apoptosis, it should be pointed out that, if a surfactant has haemolytic properties, it should be considered as a chaotropic agent, *i.e.* a compound which causes membrane disorder. Compounds with a chaotropic impact on red blood cell membranes possibly also exert a similar influence on the membranes of other types of cells. At concentrations over 10^{-4}M mixed micelles containing surfactants and natural lipids may form and the membrane be solubilised. This physico-chemical process can only occur when the surfactants are found in the membrane in sufficiently high concentrations. Molecules A, B and C have similar chemical structures, their HLB values are very close to each other (see Fig. 1), and their response to membrane phospholipids are identical which accounts for their necrotic effect.

Whenever a surfactant is used in concentrations not high enough to induce total cell necrosis, resistant cells become subject to apoptosis and develop the physiologic characteristics of apoptosis during the two-day culture. Unlike necrosis, apoptosis has a biochemical origin. Yet, no necrosis will occur if the concentration of the surfactant penetrating the membrane is not high enough to generate mixed micelles. Under these conditions the surfactants interfere with the phospholipid bilayer thus inducing several enzymatic mechanisms. Consequently an apoptotic signal is triggered in the cells. Such concentration dependent phenomena were observed for surfactants D, E and F, at levels of up to 10^{-4}M. We postulate that for these molecules the hydrophobic tails are not long enough to produce mixed micelles with the natural phospholipids. Except for surfactant E the HLB values of these compounds are indeed higher than those of surfactants G, H and I. Surfactant E comprises a fluorinated hydrophobic tail, more oleophilic than the hydrogenated analogue. Surfactants D, E and F may either enter and disturb the membrane inducing an enzymatic activity (such as that of sphingomyelinase) or, more probably, penetrate into the cell and trigger an apoptotic signal *via* the mitochondria (Château et al., 1996; Karasavvas et al., 1996).

Surfactant structure and toxicity

Surfactants A and B, which have no impact on cells, feature high HLB values (see Fig. 1). Their water solubility prevents them from entering the lipid bilayer of cell membranes. Compound C has a chemical structure characterized by a long hydrophobic tail and a bulky hydrophilic head, which enables it to remain in the phosphobilayer without disturbing it. It will not penetrate into the cell, and presents therefore no toxic effect. The results we have obtained with other surfactants and those reported previously (Leblanc et al., 1985; Maugras et al., 1989) indicate that a chain-length ranging from 12 to 18 carbon atoms allows the surfactants to incorporate smoothly with the

lipids of the cell membrane. This is the reason why no cell death is observed with this type of compounds.

We have previously conducted toxicity studies with surfactants of various chemical structures without β-lactam rings (Maugras et al., 1989; Selve et al., 1991; Lepercq, 1994) which exhibit patterns of toxicity similar to those described here. This indicates that the β-lactam structure had no particular effect on cell toxicity.

Conclusion

The surfactants studied in this work fall in three categories depending on their toxicity for living cells: non-toxic, inducing apoptosis, or inducing necrosis. The toxicity level of the two latter depends on their structure and their concentration in the culture medium. Non-toxic surfactants can be used in intense cell culturing processes as anti-foam agents; moreover, they facilitate the uptake of nutrients by lowering the interfacial tension between the cell membrane and the culture medium.

Our investigations show that for toxic surfactants apoptosis is induced in the cells after their insertion into the cell membrane. This process occurring at low surfactant concentrations seems to trigger the apoptotic signal inside the cell by transduction. At higher concentrations the disrupture of the cell membrane *via* mixed micelle formation between surfactants and membrane phospholipids leads to cell necrosis (detergent effect). The use of surfactants in biological media, e.g. for lipofection procedures, should therefore be preceded by a thorough study of their amphiphilic properties.

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