Complement activation by polyethoxylated pharmaceutical surfactants: Cremophor-EL, Tween-80 and Tween-20

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Immunosafety analysis of pharmaceutical surfactants is an important step in understanding the complex mechanisms by which they induce side effects in susceptible patients. This paper provides experimental evidences that polyethoxylated surfactants, Cremophor-EL and Tween-80, also known as Polysorbate-80, activate the complement system in vitro, in normal human serum and plasma. They appeared to be more efficient reactogens than their structural homolog, Tween-20. Cremophor-EL and Tween-80 promoted the generation of biologically active complement products, C3a, C5a and C5b-9. Consistently, Paclitaxel and Taxotere (Docetaxel), pharmaceuticals formulated in Cremophor-EL and Tween-80, activated the complement system in similar extent. Moreover, comparison of serum reactivity against the drug-loaded and drug-free formulations exhibited a significant linear correlation. Taken together, these results are consistent with the hypothesis that therapeutic side effects, such as acute hypersensitivity and systemic immunostimulation, caused by intravenous nanomedicines containing polyethoxylated detergents such as Cremophor-EL and Tween-80, can be attributed to complement activation-derived inflammatory mediators.

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1. Introduction

Cremophor-EL (CrEL), Tween-80 (T80) and Tween-20 (T20) have similar structural and functional properties. Their nonionic, surface active features are due to their hydrophilic and hydrophobic moieties, polyethoxylated glycerol or sorbitol, and oleyl or lauryl groups. Combined with a solvent, these molecules tend to self-aggregate. In aqueous solutions, above the critical micellar concentration (cmc), they form colloidal particles, spherical micelles with lipophilic interior. The size of the particles varies between 5 and 25 nm depending on the actual physico-chemical properties of the medium. Due to their amphiphilic nature, they can reduce the interfacial tension of solvent systems, and disperse a variety of immiscible materials.

Medical applications of CrEL and T80 are generally recognized as relatively safe. They belong to the most extensively used pharmaceutical surfactants in various oral and parenteral delivery systems. Their application intravenously is also approved by the US Food and Drug Administration as well as the European Medicines Agency. They are used as excipients of various hydrophobic compounds, for example, vitamins, several antineoplastic and immunosuppressive agents, anesthetics and analgesics.

CrEL and T80 are not completely inert compounds. They have been reported to interact directly with phospholipid bilayers and many proteins (Hacker et al., 1981; Friche et al., 1990; Hanke et al., 2010; Christiansen et al., 2011). Thus, they can alter pharmacological properties of active ingredients and/or cause pathophysiological symptoms such as acute hypersensitivity reactions in susceptible patients (Ten Tije et al., 2003; Coors et al., 2005). These reactions comprise flushing, urticaria, pruritus, hypotension, dyspnoea or tachypnea, moreover, in more serious cases, overwhelming anaphylactoid symptoms. In general, the acute hypersensitivity induced by above nonionic detergents is thought to be independent of specific immunoglobulins, T-lymphocytes and other adaptive elements of the immune system (Coors et al., 2005). Hence, they are mostly referred to as anaphylactoid (Lorenz et al., 1977) or pseudoallergic (Szebeni, 2005) reactions.

Abbreviations: CMC, critical micellar concentration; CrEL, Cremophor-EL; EU, endotoxin unit; MBL, mannose-binding lectin; MASP, MBL-associated serine protease; T20, Tween-20; T80, Tween-80.

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Serious forms of hypersensitivity reactions have been reported in a number of medicines containing CrEL and T80, including Paclitaxel and Taxotere, and a number of other experimental drug formulations (Glen and Hunter, 1984; Kris et al., 1986; Eschalier et al., 1988; Burris et al., 1993; Bissett et al., 1993; Theis et al., 1995; Kloover et al., 2004; Montoro et al., 2000; Ardavanis et al., 2004; Price and Hamilton, 2007). Some of these adverse effects can be ethologically related to complement activation (Hüttel et al., 1980; Dye and Watkins, 1980; Szebeni et al., 1998; Loos et al., 2002; Szebeni, 2005).

Complement system is a complex, innate, humoral immune mechanism which consists of over 30 plasma proteins, protein fragments and protein complexes (Ricklin et al., 2010). In many respect, it acts as a functional interface between the innate and adaptive immunity. When the system gets stimulated by relevant activators, the ubiquitous complement components instantly actuate a proteolytic, amplifying reaction cascade. This generates opsonins (e.g. C3b and C4b), pleiotropic proinflammatory mediators (e.g. C3a and C5a) and membrane attacking, terminal complement complexes (e.g. C5b-9). These factors facilitate phagocytosis, recruitment and stimulation of white blood cells, or perforation of plasma membranes on cellular targets and microorganisms.

In this work, mostly enzyme-linked immunosorbent assays were applied to investigate the activation of serum complement by CrEL, T80 and T20, as well as Paclitaxel and Taxotere, intravenous pharmaceuticals containing CrEL and T80, respectively. Complement activation by CrEL was studied earlier in detail (Szebeni et al., 1998). The present study is the first, to our knowledge, that investigates the complement reactivity of the above micellar dispersions in a comparative manner.

2. Materials and methods

2.1. Materials

Zymosan A and nonionic detergents, Cremophor-EL (main component: polyethoxy-35 glycerol tririncinoleate), Tween-20 (polyethoxy-20 sorbitan monooleate, i.e. Polysorbate-80, or food additive E433) and Tween-20 (polyethoxy-20 sorbitan monolaurate, i.e. Polysorbate-20, or food additive E432) were purchased from Sigma–Aldrich Ltd., Hungary. Test results were confirmed by using the PyroGene assay system purchased from Lonza Inc. Samples were diluted in sterile phosphate buffered saline (pH 7.4) containing 0.27 g/l of KH$_2$PO$_4$, 2.86 g/l of Na$_2$PO$_4$ (2H$_2$O) and 8.00 g/l of NaCl. The assay was carried out in a microtiter plate, in 96-well format, according to the procedure described in the package insert. The reaction was quantified by detection of the fluorescent enzyme products by using a Victor3 1420/FLx800 microplate reader. Conventionally, results below 1.0 EU/ml refers to an essentially endotoxin-free material. In terms of the reference lipopolysaccharide, EC-6, 1 EU/ml corresponds to a concentration of <0.1 ng/ml. It is below the maximum acceptable threshold specified for infusion products by the Pharmacopeia in the United States and in the European Union. As majority of substances interfere with endotoxin tests to some degree, the level of interference was estimated by analyzing parallel probes spiked with a known amount of endotoxin.

2.2. Determination of endotoxin levels

Endotoxin contamination of CrEL, T80 and T20 was assessed by using the PyroGene assay system purchased from Lonza Inc. Samples were diluted in sterile phosphate buffered saline (pH 7.4) containing 0.27 g/l of KH$_2$PO$_4$, 2.86 g/l of Na$_2$PO$_4$ (2H$_2$O) and 8.00 g/l of NaCl. The assay was carried out in a microtiter plate, in 96-well format, according to the procedure described in the package insert. The reaction was quantified by detection of the fluorescent enzyme products by using a Victor3 1420/FLx800 microplate reader. Conventionally, results below 1.0 EU/ml refers to an essentially endotoxin-free material. In terms of the reference lipopolysaccharide, EC-6, 1 EU/ml corresponds to a concentration of <0.1 ng/ml. It is below the maximum acceptable threshold specified for infusion products by the Pharmacopeia in the United States and in the European Union. As majority of substances interfere with endotoxin tests to some degree, the level of interference was estimated by analyzing parallel probes spiked with a known amount of endotoxin.

2.3. Characterization of dispersions by dynamic light scattering

Assessment of the diameter and dispersity of micellar particles was deduced from the results of time-dependent fluctuations in the scattering light intensity by using the model by Stokes, Einstein and Smoluchowski (Einstein, 1905; Smoluchowski, 1906). The value of Z-average diameter is also referred to as harmonic intensity-weighted average hydrodynamic diameter. In general, PDI (i.e. polydispersity index) values below 0.1 reflect a good monodispersity. All measurements were performed on a Zetasizer Nano-S device (Malvern Instruments Inc., UK) containing a 4 mW HeNe laser operating at a wavelength of 633 nm, and an avalanche photodiode detector positioned at an angle of 173°. Three replicate specimens were dissolved in particle-free distilled water at the final concentration of 0.5% (v/v). This concentration is at least 8-fold higher than the cmc reported by the supplier. Samples were placed into disposable polystyrene cuvettes (Sarstedt Inc., Germany) and held at 37 °C during the analysis. Measurements were conducted three times with fifteen sub-runs. Results were calculated by means of the Dispersion Technology Software version 5.10 (Malvern Instruments, UK).

2.4. Preparation of test formulations

Surfactants and medicines were incubated at 37 °C, above the temperature of their solid to liquid phase transition, and diluted by agitation in sterile phosphate buffered saline (pH 7.4) containing 0.27 g/l of KH$_2$PO$_4$, 2.86 g/l of Na$_2$PO$_4$ (2H$_2$O) and 8.00 g/l of NaCl. We expressed concentrations of detergents in volume percentage, because concentration of two key pharmaceutical formulations, Paclitaxel and Taxotere, is defined mostly in this unit (Rowinsky and Donehower, 1991; Hart and Acott, 2010). The approximate density of CrEL (1.05–1.06 g/ml), T80 (1.06 g/ml) and T20 (1.1 g/ml) is comparable, thus, 1% (v/v) of these detergents is equivalent to 10.5–11.0 mg/ml.

2.5. Preparation of whole blood, serum and plasma

Human blood was drawn from healthy, Caucasian volunteers by venipuncture of the median cubital vein at the elbow. For preparation of serum samples, the whole blood was allowed to clot at room temperature, centrifuged at 3500g for 7 min. For plasma specimens, blood was collected in heparin-containing tubes (VAC-UTE® Lithium Heparin blood collection tubes, Greiner), centrifuged at 2000g for 7 min. Subsequently, the supernatants, serum and plasma, were collected, and used up freshly, or aliquoted and...
stored at −70 °C. Frozen specimens were thawed at 37 °C and kept on ice until use. Our procedures were conducted in accordance with international guidelines. The applications and operating protocols were approved by the research ethics board of Semmelweis University (reference of approval: TUKEB 142/2008).

2.6. Measurement of complement activation

Human blood, serum and plasma obtained from healthy volunteers were randomly selected. Samples were not tested for complement defect prior to the experiments. Complement deficiency was not detected or suspected. This was attributed to the low sample number and the fact that complement defects are even more frequent in the population of healthy volunteers than among patients belonging to certain disease groups (e.g. infectious, inflammatory, autoimmune and some organ diseases).

The specimens were exposed to test reagents at the volume ratio of 3:1 (Szebeni et al., 1998). Typically, 15 µl of serum, plasma or blood was mixed with 5 µl of test probe in Eppendorf tubes which were then incubated at 37 °C for the time period defined in the description of the experiments. Samples were placed on ice, and complement reactions were stopped by 980 µl of specimen diluents, provided by the supplier, completed with 10 mM EDTA. Reaction markers of complement activation were quantitated by enzyme-linked immunosorbent assays. The reagent kits for C3a-desArg and SC5b-9 were purchased from Quidel Inc., while the C5a-desArg test was supplied by BD Biosciences Inc. These products had been developed to optimize the sensitivity by measuring relevant, relatively stable derivatives (e.g. C3a-desArg, C5a-desArg and SC5b-9) instead of the primary complement products showing shorter half-life (e.g. C3a, C5a and SC5b-9). Absorbance values at 450 nm were measured by a Victor3 1420/Flx800 microplate reader. Concentration of analytes was determined in the linear range of calibration curves. Reproducibility of results was confirmed by testing CrEL, T80 and T20 samples derived from independent sources: Sigma (data shown) and Caesar & Loretz (data not shown).

2.7. Statistical analysis

Kolmogorov–Smirnov test was conducted to evaluate the normality assumption of the data sets. To assess statistical significance, one-way ANOVA followed by Dunnnett’s post-hoc evaluation, Student’s paired t-test, and Pearson’s two-tailed correlation analysis were used. At p < 0.05, the difference between two groups was considered to be statistically significant. A test material induced complement activation, when the concentrations of the studied surfactants. Samples were measured above the cmc, at 0.5% (v/v) and 37 °C. Levels of complement activation products were measured by using enzyme-linked immunosorbent assays monitoring the analytes produced at different stages of the complement activation. Each detergent induced significant production of C3a-desArg relative to the vehicle group (p < 0.05) (Fig. 2A). The reactogenicity of detergents and Zymosan A, used at 100 µg/mL as positive control, was comparable. Furthermore, two surfactants, CrEL and T80, stimulated a significant increase of C5a-desArg and SC5b-9 (p < 0.01), while T20 caused only a minor elevation of these reaction markers (p > 0.05) (Fig. 2B and 2C). These effects and differences were similar in experiments in which the samples were incubated for 45 min in the presence of an independent series of detergents (data not shown).

Kinetics and dose–response relationship of the test surfactants were assessed by measuring SC5b-9, as, in our previous studies, this reaction marker exhibited the strongest relative response over the control. After 10 min at 37 °C, increased levels of SC5b-9 were detected in sera treated with CrEL and T80 (Fig. 3A). Half maximal responses were shown between 10 and 30 min, and a steadily increasing dose–response curve was registered in the concentration range of 0.1–2.0% (v/v) (Fig. 3B). In contrast, T20 was significantly less efficient at generating SC5b-9 in these experiments (p < 0.001).

Fig. 4 represents experiments wherein complement activation by CrEL, T80 and T20 was assessed in fresh specimens: in serum, plasma and whole blood. In these studies, the reactogenicity profile of detergents was similar to those observed previously in cryopreserved sera. CrEL and T80 turned out to be complement activating in fresh serum and plasma, while T20 was significantly less.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>% Intensity</th>
<th>Z-average diametera (nm)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor-EL</td>
<td>100</td>
<td>13.05 ± 0.03b</td>
<td>0.03 ± 0.012</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>100</td>
<td>13.66 ± 0.08</td>
<td>0.04 ± 0.005</td>
</tr>
<tr>
<td>Tween-80</td>
<td>100</td>
<td>10.08 ± 0.02</td>
<td>0.06 ± 0.010</td>
</tr>
<tr>
<td>Taxotere</td>
<td>100</td>
<td>11.05 ± 0.01</td>
<td>0.05 ± 0.007</td>
</tr>
<tr>
<td>Tween-20</td>
<td>100</td>
<td>7.89 ± 0.01</td>
<td>0.06 ± 0.017</td>
</tr>
</tbody>
</table>

a Determined by using a Zetasizer Nano-Z instrument, using the Stokes–Einstein model. The viscosity of the solution taken as ρ(H2O) = 0.686 cP.

3.2. Analysis of dispersions by dynamic light scatter

Dispersions of CrEL, T80 and T20 were characterized in water-based solutions at the final concentration of 0.5% (v/v). Distribution of percentage intensity values generated single, unimodal histograms with polydispersity indices lower than 0.1 (Table 1 and Fig. 1). These factors indicated that the reagents at the test conditions formed monodisperse, micellar, colloidial systems. The hydrodynamic diameters, Z-average diameters, were ranging from 7.89 to 13.05 nm. The lowest and highest values were produced by T20 and CrEL, respectively. Their difference was about 65%. On the other hand, the difference between CrEL and T80 was about 30%. These differences between the peak intensities were statistically significant (p < 0.0001). Likewise, Paclitaxel and Taxotere formed monodisperse, micellar systems due to their main ingredients, CrEL and T80, respectively. Their micelles appeared to be bigger than that of CrEL and T80, however, these differences were not significant.

3.3. In vitro complement activation

First, the influence of CrEL, T80 and T20 on serum complement was investigated at the final concentration of 1.0% (v/v). Randomly selected normal human sera were exposed to the surfactants for 15 min at 37 °C. Levels of complement activation products were measured by using enzyme-linked immunosorbent assays monitoring the analytes produced at different stages of the complement activation. Each detergent induced significant production of C3a-desArg relative to the vehicle group (p < 0.05) (Fig. 2A). The reactogenicity of detergents and Zymosan A, used at 100 µg/mL as positive control, was comparable. Furthermore, two surfactants, CrEL and T80, stimulated a significant increase of C5a-desArg and SC5b-9 (p < 0.01), while T20 caused only a minor elevation of these reaction markers (p > 0.05) (Fig. 2B and 2C). These effects and differences were similar in experiments in which the samples were incubated for 45 min in the presence of an independent series of detergents (data not shown).
effective. Similar reaction pattern was seen in whole blood, though, the group differences were not significant in this case with the exception of Zymosan A used as positive control.

Finally, the capacity of 14 intravenous antineoplastic drugs to activate complement was determined. As shown in Fig. 5A, the groups of non-micellar drug formulations (Carboplatin, Doxorubicin, Endoxan, Fluourouracil, Gemzar, Irinotecan, Mytomycin-C, Oxaliplatin, Platidium, Vinblastin, Eributux and Herceptin) failed to elicit a significant SC5b-9 response. On the other hand, SC5b-9 production was stimulated significantly \((p < 0.001)\) by micellar taxane formulations, Paclitaxel and Taxotere, containing CrEL and T80, respectively. Subsequent analysis of the same sera revealed that drug-loaded and drug-free CrEL and T80 produced a comparable complement activation (Fig. 5B). Moreover, serum reactions caused by drug-loaded and drug-free micellar formulations exhibited a significant correlation \((p < 0.05)\) (Fig. 5C). These effects and correlations were reproducible in experiments using an independent series of detergents (data not shown).

4. Discussion

The main goal of this study was to increase the understanding of infusion-related complement activation by micellar drug carriers. Therefore, we have investigated complement reactions stimulated by CrEL, T80 and T20 as well as Paclitaxel and Taxotere, pharmaceuticals containing CrEL and T80, respectively. This experimental system permitted a simple, comparison-based analysis of micellar formulations to explore how their molecular features and drug-loading determine their complement reactogenicity.

First, endotoxin levels of CrEL, T80 and T20 were measured by using a commercial diagnostic assay based upon endotoxin binding to recombinant clotting factor C, a protein genetically engineered by virtue of the endotoxin-recognizing component identified in the horseshoe crab, *Limulus polyphemus* (Ding and Ho, 2010). CrEL, T80 and T20 proved to be endotoxin-free. Besides, the colloidal systems of test materials formed in aqueous environment above the cmc were analyzed by dynamic light scatter measurements. In each sample, the parameters demonstrated a good monodispersity in the size range of small micelles. The results were consistent with previously reported data (Dimitrova and Leal-Calderon, 2000).

In spite of the fact that the strength of complement reactions varied considerably depending on the individual susceptibility of sera, the results of our studies demonstrated, that CrEL and T80 activated the complement system in cryopreserved, normal human sera at physiological temperature. CrEL and T80 promoted

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**Fig. 1.** Histograms representing micellar size distribution of drug-free and drug-loaded pharmaceutical surfactants: Tween-20 (Panel A-B), Cremophor-EL vs. Paclitaxel (Panel A), and Tween-80 vs. Taxotere (Panel B). Samples were diluted to 0.5% (v/v). Dynamic light scatter measurements were conducted at 37 °C. In each group, three parallel samples were measured three times with fifteen sub-runs.

**Fig. 2.** Complement activation by Cremophor-EL, Tween-80 and Tween-20 at 1% (v/v, final concentration) in normal human sera. Serum levels of C3a-desArg (A), C5a-desArg (B) and SC5b-9 (C) were determined after incubation for 15 min at 37 °C. Zymosan A was used at 100 μg/ml as positive control. Phosphate buffered saline (pH 7.4) was used as vehicle. The concentration of analytes is represented by dots and mean ± SEM. \((n = 4; * p < 0.05; ** p < 0.01; *** p < 0.001).\)
significant production of C3a, an anaphylatoxin generated upon the cleavage of C3 protein by C3 convertase complexes. Moreover, these events were shown to culminate subsequently in the terminal complement pathway: activation of C5 convertases, significant production of C5a, a further anaphylatoxin, as well as SC5b-9, a lytically inactive form of membrane attack complexes. SC5b-9 is generated by association of the nascent C5b-9 with S-protein (vitronectin) in the absence of appropriate target cell membranes (Bhakdi et al., 1979a). As the comparison of analytes revealed that SC5b-9 was the most sensitive measure reflecting activation of the whole complement cascade, SC5b-9 was predominantly used as reaction marker in subsequent studies.

Reactogenicity of Crel and T80 was reproducible in fresh serum and plasma. Moreover, a similar reaction pattern was observed in whole blood, although, the response was interfered in some extent by the moderate cytolysis and hemolysis caused by the detergents at the test concentration (Bhakdi et al., 1979b), and by the partial depletion of C5b-9 and SC5b-9 in consequence of their membrane- and receptor-binding, respectively (Hammer et al., 1975; Bhakdi et al., 1979a; Røger et al., 1995; Biesecker, 1990).

At 100-fold dilutions of Crel and T80, half-maximal complement activation was reached within 30 min of incubation, indicating that the initiation and progression of this humoral immune reaction is relatively slow. A steadily increasing production of SC5b-9 was observed in the concentration range of 0.1–2.0% (v/v). These studies demonstrated that complement activation is proportional to the incubation time and surfactant concentration. As the efficient concentrations were found to be above the cmc, formation of micelles seems to be a critical prerequisite of complement activation by the surfactants.

The effect of the presence and absence of surfactants was tested in infusion medicines as well. The comprehensive analysis involved 12 essentially detergent-free intravenous products. These aqueous, non-colloidal solutions of small active compounds or biologicals were shown to be virtually ineffective (p > 0.05), unable to cause complement activation. Consistent complement reactions could be provoked only with those two pharmaceuticals, Paclitaxel and Taxotere, in which the relatively small active compounds were formulated in nonionic, micellar excipients. Paclitaxel and Taxotere are semi-synthetic taxane derivatives formulated in 50% (v/v) of nonionic detergents, Crel and T80, respectively (Rowinsky and Donehower, 1991; Hart and Acott, 2010). Besides the surfactants, the mixture contains ethanol, 50% and 13%, respectively, to solubilize the system at room temperature. When Paclitaxel and Taxotere were diluted to contain only 1% (v/v) of detergents, they could be provoked only with those two pharmaceuticals, Paclitaxel and Taxotere, in which the relatively small active compounds were solubilized in infusion medicines as well. The comprehensive analysis involved 12 essentially detergent-free intravenous products. These aqueous, non-colloidal solutions of small active compounds or biologicals were shown to be virtually ineffective (p > 0.05), unable to cause complement activation. Consistent complement reactions could be provoked only with those two pharmaceuticals, Paclitaxel and Taxotere, in which the relatively small active compounds were formulated in nonionic, micellar excipients. Paclitaxel and Taxotere are semi-synthetic taxane derivatives formulated in 50% (v/v) of nonionic detergents, Crel and T80, respectively (Rowinsky and Donehower, 1991; Hart and Acott, 2010). Besides the surfactants, the mixture contains ethanol, 50% and 13%, respectively, to solubilize the system at room temperature. When Paclitaxel and Taxotere were diluted to contain only 1% (v/v) of detergents, they were able to elicit a significant complement response in human serum. Responses against the drugs and detergent-containing placebos proved to be virtually identical. Moreover, the individual susceptibility of sera for drug and placebo formulations exhibited a significant correlation. The serum reaction was generated irrespectively of whether the active compound was present or absent in the formulation. These results demonstrate that Paclitaxel and Taxotere activate complement solely due to the effect of micellar drug carriers, Crel and T80.
Fig. 5. Complement reactogenicity of intravenous medications and pharmaceutical detergents. In Panel A, normal human sera were exposed to pharmaceuticals formulated either in water essentially free of any detergents (Carboplatin, Doxorubicin, Endoxan, Fluorouracil, Gemzar, Irinotecan, Mitomycin-C, Oxalaplatin, Platinium, Vinblastin, Erbitux, Herceptin), or dispersed by using pharmaceutical surfactants (Paclitaxel and Taxotere). The medicines were used uniformly at a 40-fold dilution of the package units after appropriate reconstitution. At this dilution Paclitaxel and Taxotere contain ca. 1% (v/v) of detergents. Panel B shows results of serum complement studies obtained with drug-free placebo controls of Paclitaxel and Taxotere. In these experiments the same sera were consecutively exposed to the micellar drug carriers: Cremophor-EL, Tween-80 and Tween-20 at 1% (v/v). In Panel C, the correlations of serum susceptibilities to drug-loaded vs. drug-free formulations are shown. Data points represent relative increase of SC5b-9 derived from the results shown in Panel A and B. In these experiments (Panel A, B and C), sera were treated with test materials for 15 min at 37 °C. Zymosan A was used at 100 μg/ml as positive control. Error bars represent SEM. (n = 17; "p < 0.05; ""p < 0.01; """"p < 0.001).

Despite of numerous studies and assumptions, it has not yet been clarified precisely which direct and/or indirect effects of pharmaceutical surfactants are responsible for the complement activation in the fluid phase of human blood. In our study, a significant production of C3a was shown in response to each polyethoxylated nonionic detergent, including T20. This proves complement reactions which are effectively propagated towards the activation of C3 convertase complexes. Polyethoxylated detergents, similarly to pegylated nanoparticles, comprise terminal hydroxyl-polyethoxylene segments. Therefore, the fact that each surfactant was effective at this stage of complement activation is consistent with previous results obtained with hydroxyl polyethoxylene-containing nanoparticles such as poloxamers (Moghimi et al., 2004), polyethylene-glycols (Hamad et al., 2008), pegylated liposomes and pegylated carbon nanotubes (Moghimi et al. 2010). Moreover, it is in accordance with a current concept by Moghimi et al. (2011) suggesting that some polyethoxylated/pegylated nanoparticles can be recognized by mannosese-sensing pattern recognition elements. Namely, on the multiple ‘OH–CH2–CH2–O–’ backbone segments which are displayed on mannan polymers as well as on the surface of micelles and vesicles of poloxamer block co-polymers, but also on the micelles of polyethoxylated surfactants such as CrEL, T80 and T20. They can serve as a putative docking site for MBL (Moghimi et al., 2011; Moghimi et al., 2004). In addition, the nucelophilic hydroxyls in the terminal hydroxyl polyethoxylene segments are also favorable for complement activation by allowing thioester group-mediated fixation of nascent C3b. This way, surface-bound C3b molecules can synergize with mannan binding lectins to induce the complement reaction cascade by facilitating assembly and activation of C3 convertases and, subsequently, C5 convertase complexes. Our data demonstrate that CrEL and T80 are significantly more effective than T20 in activation of C5 convertases. Based on structural differences, this suggests that, compared to saturated fatty acid moieties of T20, unsaturated fatty acids of CrEL and T80 have a differential impact on the progression of the complement activation. They favor assembly and activation of C5 convertases.

Taken together, our studies have been conducted to investigate in vitro complement reactogenicity of pharmaceutical surfactants. The results presented here reinforces that, in intravenous medicines, CrEL and T80 represent a considerable risk for complement activation and immunological side effects such as acute hypersensitivity and systemic immune reactions (Kris et al., 1986; Szebeni et al., 1998; Szebeni et al., 2001; Burris et al., 1993; Ten Tije et al., 2003). In the clinical practice, pathological manifestations of adverse infusion reactions are attenuated routinely by high dilution and slow rate of infusions (van Zuylen et al., 2000), and package-insert-specified, prophylactic premedication protocols (Raisch et al., 2011). Lately, as an alternative approach to premedication, a detergent-free, protein-nanoparticle formulated paclitaxel with improved safety profile has been developed (Desai et al., 2006; Henderson and Bhatia, 2007). The ultimate goal of these studies, procedures and developments is to gain insight into the immunological events responsible for adverse reactions, and/or, to improve the safety and efficacy of the micellar nanomedicines in question.

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