Journal of Controlled Release 195 (2014) 2–10

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Features of complement activation-related pseudoallergy to liposomes with different surface charge and PEGylation: Comparison of the porcine and rat responses

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ARTICLE INFO

Article history:
Received 16 May 2014
Accepted 8 August 2014
Available online 19 August 2014

Keywords:
Nanomedicines
Anaphylaxis
Adverse drug effects
Anaphylatoxins
Pseudoallergy
CARPA

ABSTRACT

Pigs are known to provide a sensitive model for studying complement (C) activation-related pseudoallergy (CARPA), a hypersensitivity reaction to liposomal and many other nanomedicines that limits their clinical use. The utility of rats as a CARPA model has, however, not been analyzed to date in detail. The present study compared the two models by inducing CARPA with i.v. bolus injections of two reactogenic liposomes that differed from each other in surface properties: one was AmBisome, a strong anionic, free-surface small unilamellar liposome (SUV), while the other was neutral, polyethylene glycol (PEG)-grafted SUV wherein the 2 kDa-PEG was anchored to the membrane *via* cholesterol (Chol-PEG). Both in pigs and rats AmBisome caused significant consumption of C3, indicating C activation, along with paralleling massive changes in blood pressure, white blood cell, platelet counts and in plasma thromboxane B2 levels, indicating CARPA. These processes were similar in the two species in terms of kinetics, but significantly differed in the doses that caused major hemodynamic changes (~0.01 and ~22 mg phospholipid (PL)/kg in pigs and rats, respectively). Pigs responded to AmBisome with pulmonary hypertension and systemic hypotension, and the reaction was not tachyphylactic. The major response of rats was systemic hypotension, leukopenia followed by leukocytosis, and thrombocytopenia. Chol-PEG liposomes caused severe reaction in pigs at 0.1 mg/kg, while the reaction they caused in rats was mild even at 300 mg PL/kg. Importantly, the reaction to Chol-PEG in pigs was partly tachyphylactic. These observations highlight fundamental differences in the immune mechanisms of porcine and rat CARPA, and also show a major impact of liposome surface characteristics, determining the presence or absence of tachyphylaxis. The data suggest that rats are 2–3 orders of magnitude less sensitive to liposomal CARPA than pigs; however, the causes of these differences, the PEG-dependent tachyphylaxis and the massive reactivity of Chol-PEG liposomes remain unclear.

1. Introduction

One potential problem with the intravenous use of controlled drug delivery systems, such as liposomal drugs, is their recognition by the immune system as harmful particles. The response is a hypersensitivity, or infusion reaction, recently described as complement (C) activation-related pseudoallergy (CARPA) [1,2]. Because of its potential fatal outcome, the phenomenon is considered as a safety issue in nanopharmacotherapy [3–8], whose assessment was recently recommended by the European Medicines Agency as a preclinical immune toxicity test in the development of (generic) liposomal drugs [9]. However, at present, there is no standard test, or a validated battery of testing procedures for evaluating the CARPAgenic activity of i.v. administered nanomedicines, similar to the hemocompatibility tests mandated by regulatory agencies for the human application of medical devices (*e.g.*, endovascular grafts, shunts, rings, patches, heart valves, balloon pumps, stents, pacemakers, hemapheresis filters) [7,10].

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http://dx.doi.org/10.1016/j.jconrel.2014.08.009
0168-3659/

To date, C activation by nanomedicines and its adverse consequences have been studied either *in vitro*, by measuring C cleavage products in human or animal sera, or *in vivo*, by injecting the test materials in animals i.v., mostly in bolus, and monitoring the ensuing hemodynamic, hematological and biochemical changes that mimic the symptoms of severe human hypersensitivity reactions [7,11]. However, these assays use a large variety of conditions and endpoints which have not been standardized yet. Moreover, the *in vivo* assays critically depend on the animal model used, as the sensitivity of CARPA seems to substantially differ in different animal species [12].

The purpose of the present study was to contribute to the development of a standardized animal assay for CARPA by comparing the reactions in pigs and rats, *i.e.*, two species wherein liposomes were shown to have significant hemodynamic, hematological and biochemical changes. The high sensitivity of pigs to liposomal CARPA, and, hence, unique use of this animal as a CARPA model, has been amply demonstrated and discussed in previous studies [1,13–17]. The major adverse physiological effects of liposomes in rats have also been described earlier, however, this species was not considered as a standardizable animal model for nanomedicine-induced hypersensitivity reactions, mainly because the focus of previous studies – mostly by Rabinovici et al. in the 1990s – was highlighting the adverse effects of hemoglobin-containing liposomes [18–27], rather than the use of rats as an immune toxicity test. Thus, the present study fills a gap in the search for methods in immune toxicology. A further goal of our experiments was to explore the impact of liposome surface charge and PEGylation on CARPA. These surface properties have previously been suggested as major players in C activation and CARPA [16]. The vesicles that we used to trigger CARPA represent the extremes of two frequently applied modifications on the liposome phospholipid bilayer surface: the introduction of negative charge (AmBisome) and the conjugation of a PEG polymer to the surface (Chol-PEG: a neutral PEGylated liposome).

2. Materials and methods

2.1. Materials

Commercial AmBisome (Gilead, Astellas Pharma US, Inc.) and Doxil were obtained from a pharmacy in Budapest, Hungary. AmBisome consists of soy phosphatidylcholine (HSPC), cholesterol (Chol), distearoylphosphatidylglycerol (DSPG), α-tocopherol and disodium succinate hexahydrate buffer in the following weight ratio: 50:213:52:84:0.64:27, respectively. From among the lipids used to prepare PEGylated liposomes dipalmitoylphosphatidylcholine (DPPC) and 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate (2K-mPEG-DSPE) were from Avanti Polar Lipids Inc. (Alabaster, USA), 1,2-distearoyl-SN-glycero-3-phosphatidyl-ethanolamine (DSPE), cholesterol-2K and 5K-PEG conjugates (2K-PEG-Chol, 5K-PEG-Chol) were obtained from NOF Europe (Grobbendonk, Belgium) and Chol was from Sigma-Aldrich (The Netherlands). All lipids were of pharmaceutical or highly pure grade (purity ≥97%) and were used without further purification. Phosphate buffered saline (PBS) was purchased from B. Braun, Melsungen (Germany). Zymosan was from Sigma Chemical Co. (St. Louis, MO). Human serum samples from healthy volunteer donors were obtained through an institutionally approved phlebotomy protocol, and were stored at −70 °C until use.

2.2. Methods

2.2.1. Preparation and characterization of liposomes

Liposomes were prepared using a film extrusion method, as detailed earlier [28]. The lipids were dissolved in ethanol in molar ratios as described in Table 1 and a thin film was created by rotary evaporation at 70 °C. The film was hydrated in PBS and the resulting coarse dispersion was downsized by multiple extrusions through polycarbonate filter membranes with pore size of 100 nm. The size and size-distribution (polydispersity index, PDI) of liposomes were determined by dynamic light scattering (DLS) with a Malvern ALV CGS-3 system (Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom), at a scattering angle of 90° at 25 °C. Samples were diluted 200 times in PBS before measurement. The zeta-potential of liposomal formulations was measured by Laser Doppler Micro-Electrophoresis, using a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom). Samples were diluted 1:100 in 10 mM HEPES buffer.

2.2.2. In vitro assays in human and animal serum

2.2.2.1. SC5b-9 ELISA. Whole blood samples of 3 healthy volunteers were collected in 10.0 mL BD Vacutainer® silicon coated glass serum tubes with no additives (BD, Franklin Lakes, NJ, USA). Blood samples were allowed to clot at room temperature and subsequently centrifuged at 3000 rpm for 5 min to collect serum. Serum samples were aliquoted and stored at −20 °C. Frozen samples were rapidly thawed at 37 °C and kept on ice until use. Complement activation was assessed by MicroVue SC5b-9 Plus ELISA kits (Quidel Co., San Diego, CA, USA). Serum from healthy volunteers was incubated with the diluted liposomal formulations (4:1) in duplicate for 30 min at 37 °C. In a typical experiment we mixed 10 μL of liposomes with 40 μL of serum in Eppendorf tubes, which were then incubated in a shaking water bath (shaking rate of 80 rpm). After incubation the samples were diluted 20-fold in the "sample diluent" of the kit (10 mM EDTA, 25 mg/mL bovine serum albumin, 0.05% Tween 20, and 0.01% thimerosal, pH 7.4) and 100 μL aliquots from this mixture were applied into the wells of the ELISA plate. The assays were performed according to the manual supplied with the kit. The absorbance was measured using a Wallac 1420 Victor 96-well plate reader (PerkinElmer, Waltham, MA, USA) at 450 nm. SC5b-9 concentrations were calculated using a linear curve fit.

2.2.2.2. Hemolytic C assay. The total C activation was determined using the classical hemolytic C assay (CH50). A fixed volume of antibody-sensitized sheep red blood cells (SRBCs) was added to serum with appropriate dilution. After incubation, the mixture was centrifuged, and hemolysis was quantified by measuring the absorbance of the hemoglobin released into the supernatant at 540 nm.

2.2.2.3. TXB2 assay. Plasma TXB2 (the stable metabolite of TXA2) levels were measured with a commercially available ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA).

Table 1
Characteristics of liposomes.

Name	Character	Lipid composition	Mole ratios	Size (nm)	PDI	Zeta potential (mV)
AmBisome	Anionic, no PEG	HSPC/Chol/DSPG/Vit-E/Amph-B	49:23:18:0.3:9	98	0.12	−53.5
2K-PEG-DSPE	Anionic, PEG	DPPC/2K-PEG-DSPE/Chol	62:5:33	96	0.06	−8.5
2K-PEG-Chol	Neutral-PEGylated	DPPC/DSPE/Chol/2K-PEG-Chol	62:5:28:5	97	0.05	0.49
Chol-PEG		DPPC/DSPE/Chol/5K-PEG-Chol		96	0.09	−0.3

The size, PDI and zeta potential entries represent the means of duplicate, triplicate or more determinations. Their variation was minor or negligible. Abbreviations: Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; DSPE, 1,2-distearoyl-SN-glycero-3-phosphatidyl-ethanolamine; DSPG, distearoylphosphatidylglycerol; HSPC, hydrogenated soy phosphatidylcholine; Vit-E, α-tocopherol; 2K-PEG-DSPE, 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethylene-glycol conjugate. The liposomes used for the animal experiments are bolded.

2.2.3. Animal studies

2.2.3.1. Rat protocol. Experiments were performed in male Wistar rats weighing 400–600 g (Toxicoop, Budapest, Hungary). Animals were anesthetized with thiobarbital sodium (Inactin, 120 mg/kg i.p. Byk, Germany). To facilitate respiration, a PE tubing (OD 3.0 mm) was inserted into the trachea and the rats spontaneously breathed. The left common carotid artery, the left femoral artery and vein were cannulated using PE-90 or PE-50 tubing. Following surgery a 30 min resting period was allowed before injecting the test material (liposomes or zymosan) as bolus (injection <10 s) in the left femoral vein. The amount of test material injected is given as mg PL/kg, unless otherwise indicated.

Arterial blood pressure was continuously recorded *via* the femoral artery catheter using Haemosys data acquisition and analysis system (Experimetria Ltd., Budapest, Hungary) or AD Instruments PowerLab System with LabChart Pro v6 software. Systemic arterial blood pressure (SAP) and heart rate (HR) were obtained before blood sampling. Blood samples were collected from the common carotid artery into Eppendorf tubes containing hirudin (lepirudin, Refludan) (for the CH50 assay) or into K_2-EDTA-containing Eppendorf tubes (for blood cell counting with an Abacus, Hematology Analyzer, Diatron, Budapest, Hungary). Blood was centrifuged at 1500 rpm for 10 min at 4 °C, and the plasma was stored at −80 °C until further analysis.

2.2.3.2. Pig studies. Domestic male Yorkshire pigs (20–25 kg) were sedated with Calypsol/Xilazine (10 and 2 mg/kg respectively) and then anesthetized with isoflurane (2–3% in O_2). Intubation was performed with endotracheal tubes to maintain free airways, and to enable controlled ventilation if necessary. Animals were breathing spontaneously. Respiration was monitored using a pulse-oximeter (fixed on the tail), monitoring oxygen saturation. Temperature was measured rectally and monitored by Innocare-P Anesthesia Monitor. A capnograph was connected to the tracheal tube to monitor $etCO_2$ and the respiratory rate (CAP10 Medlab, Medlabmedizinische Diagnosegerate GmbH, Karlsruhe, Germany). Surgery was done after povidone iodine (10%) sterilization of the skin. In order to measure the pulmonary arterial blood pressure (PAP), a Swan–Ganz catheter (Teleflex Medical, Research Triangle Park, NC, USA) was introduced into the pulmonary artery *via* the right external jugular vein, while SAP was measured in the femoral artery. Blood samples were taken from the left femoral vein and were collected in hirudin- or K_2-EDTA tubes. Liposomes and zymosan were injected in the animals in bolus (<10 s) *via* the left external jugular vein. As with rats, the amount of test material injected is given as mg PL/kg, unless otherwise indicated.

Hemodynamic changes were continuously monitored at 1000 Hz sampling rate, using an AD Instruments PowerLab System with LabChart Pro v6 software. From the mean PAP, SAP and HR data about

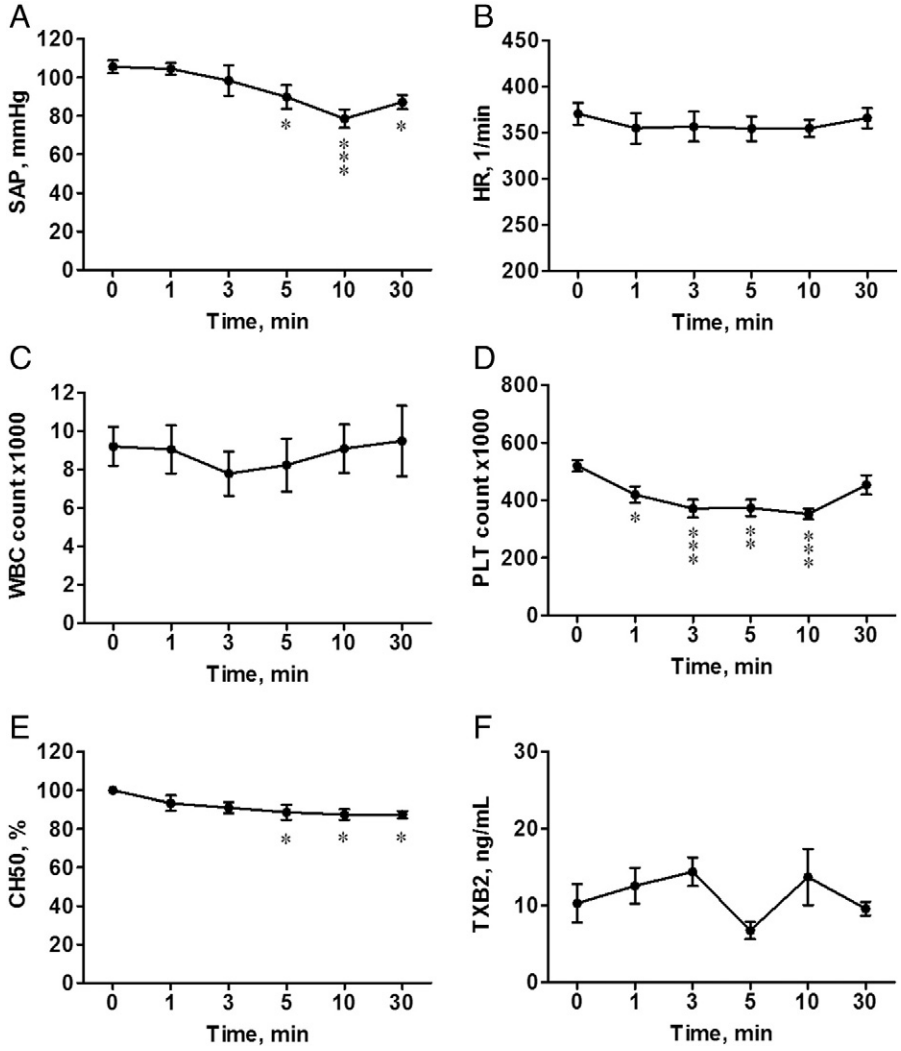

Fig. 1. Physiological changes in rats injected with AmBisome (2.2 mg/kg). Rats were injected i.v. with commercial AmBisome that was composed before the experiment according to the manufacturer's instructions. Further experimental details are described in the Methods section. Values shown are mean ± SE for 8 animals. The curves were constructed from the 0, 1, 3, 5, 10 and 30 min readings of SAP and HR after injection, as well as of other parameters were measured from blood samples taken at the same time points. *, **, ***: $p < 0.05, 0.01, 0.001$ *vs.* the time 0 value (one-way ANOVA with repeated measures), respectively.

20 s intervals were averaged and evaluated by ADInstruments LabChart Pro v6 software modules. The listed parameters were transported to Excel spreadsheet software for further analysis. The usual evaluated periods were: before the test material injection, then 20 s in every minute for 3 min, and every 5 min until the end of the reaction.

2.2.4. Statistical analysis

Changes in SAP, HR, WBC and PLT counts, as well as CH50 and TXB_2 were compared to time 0 (baseline) values. One-way ANOVA with repeated measures was performed to compare differences between time 0 and each later time point using Dunnett's post hoc test. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Liposome characteristics

Table 1 shows the composition and some structural features of the liposomes used in this study. They had nearly identical size and polydispersity, but differed in surface charge, PEG-size and anchor. In particular, AmBisome is a non-PEGylated, highly anionic liposome, DSPE-anchored 2K-PEG liposomes are anionic (but less anionic than AmBisome) PEGylated vesicles, while Chol-anchored 2K-PEG liposomes (abbreviated only as Chol-PEG liposomes) and Chol-anchored 5K-PEG liposomes are near neutral, referred as "neutral" PEGylated vesicles.

3.2. Complement activation by liposomes in normal human serum in vitro

Earlier studies have shown that both AmBisome and Chol-PEG liposomes were strong activators of C in normal human serum (NHS). In Ref. [16], for example, AmBisome caused an average of 2,7-fold rise of SC5b-9 over baseline following incubation in NHS for 30 min, with values in the 1.2–4.5-fold range ($n = 20$ different sera). Yet in another series, AmBisome caused 9.5 ± 1.1-fold rise (mean ± S.E.M., $n = 10$, unpublished data), implying substantial inter-experimental variation of the extent of C activation by AmBisome in NHS. Likewise variable, Chol-PEG liposomes in Ref. [28] caused 8–10-fold increase of SC5b-9 ($n = 12$ with values in the 4–16-range), while in a small control study carried out to test Chol-PEG and other liposomes for the present study, the activation ratio was 2.5 ± 0.1-fold ($n = 3$). In the latter study liposomes containing 2K-PEG-DSPE and 5K-PEG-Chol and zymosan led, respectively, to 0.9 ± 0.04, 0.9 ± 0.1 and 2.7 ± 0.1-fold rises of SC5b-9 over PBS control ($n = 3$), thus confirming the Chol-PEG specific C activation of PEGylated liposomes.

Based on these data, which unambiguously show the C activating capabilities of AmBisome and Chol-PEG regardless of the absolute extent of this activation, we used only AmBisome and Chol-PEG liposomes in the animal experiments described below. As

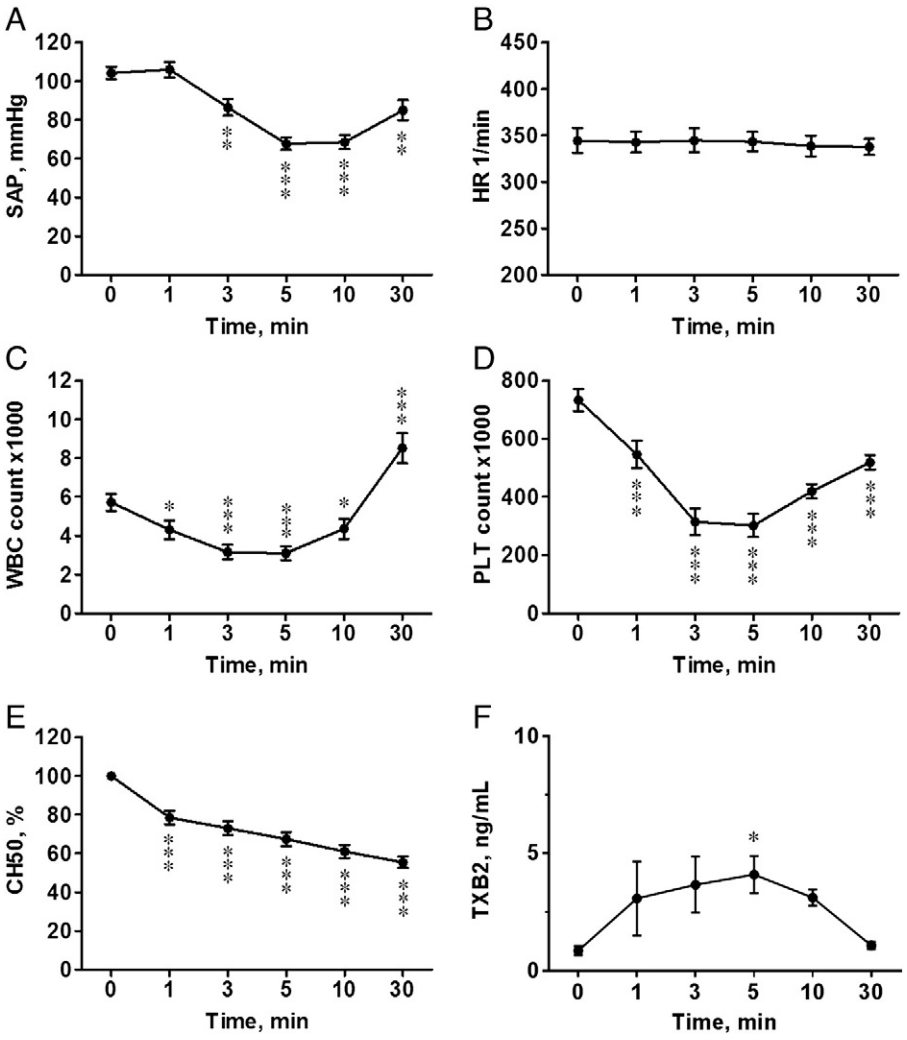

Fig. 2. Physiological changes in rats injected with 10-fold higher dose of AmBisome. Similar experiments as in Fig. 1, except that rats were injected with 22 mg PL/kg AmBisome (mean ± SE, $n = 8$).

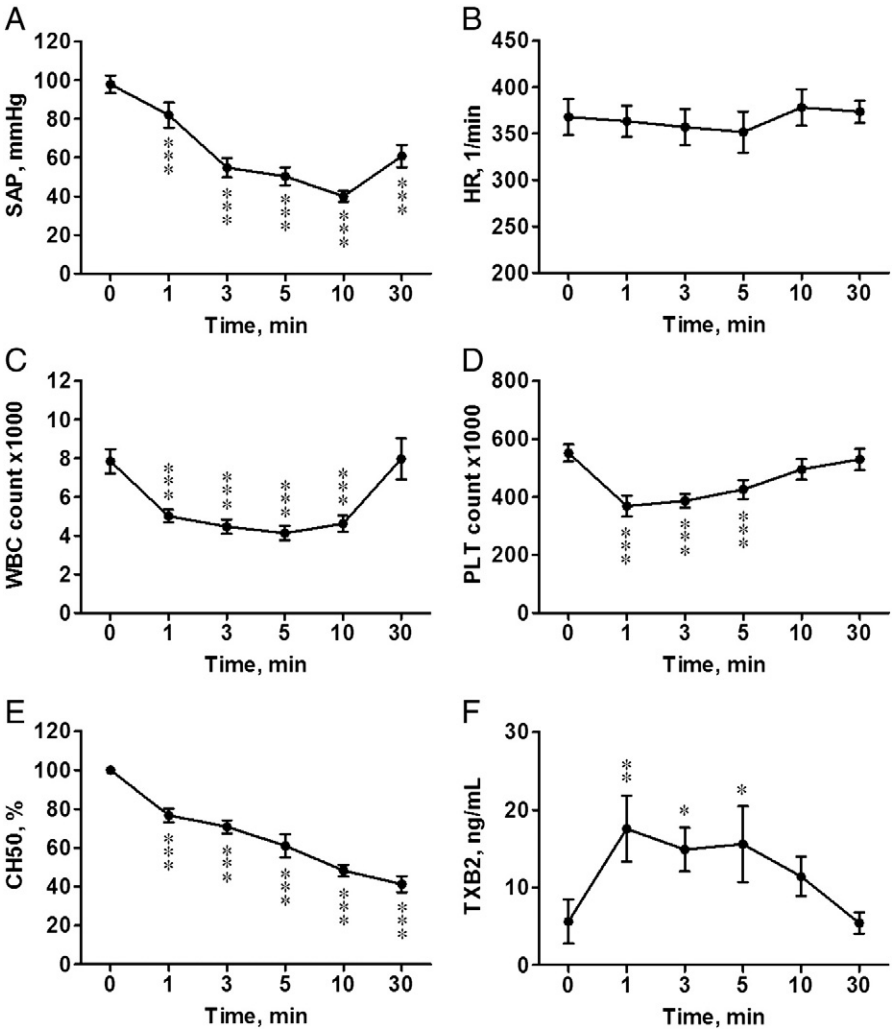

Fig. 3. Physiological changes in rats injected with zymosan. Similar experiments as in Fig. 1 except that rats were injected with 10 mg/kg zymosan (mean ± SE, n = 8).

positive control we used zymosan, and in one of the pig studies we also used liposomal doxorubicin (Doxil), a PEGylated, slightly anionic liposome that has been extensively characterized elsewhere [16,17].

3.3. Physiological changes in rats caused by Liposomes

3.3.1. Effects of AmBisome

Fig. 1 A shows that i.v. administration of AmBisome in rats at 2.2 mg PL/kg leds to a gradual decrease of SAP by about 20% at 10 min post injection, which decrease, although statistically significant, represents relatively minor change compared to the effects of higher doses (see below in Figs. 2 and 3). It is important to point out in this figure that this 20% drop of SAP was closely paralleled by simultaneous and proportionate drops in platelet (PL) counts (Fig. 1D) and plasma C hemolytic activity (Fig. 1E), suggesting direct or indirect causal relationships among these parameters. However, the HR (Fig. 1B), WBC (Fig. 1C), and plasma TXB_2 levels (Fig. 1F) were not changed relative to baseline.

Fig. 2 shows the same physiological parameters after bolus administration of a 10-fold higher (22 mg PL/kg) dose of AmBisome. Here, the SAP dropped by 40% after 5 min (Fig. 2A) which change, just as in Fig. 1, was associated with quantitatively and kinetically paralleling thrombocytopenia (Fig. 2D) and reduction of hemolytic activity (Fig. 2E). At its high dose, however, we also observed significant (50%) initial leukopenia at 5 min, switching to leukocytosis by 10 min (Fig. 2C). The HR (Fig. 2B) did not change, and plasma TXB_2 rose minimally (Fig. 2F).

3.3.2. Effects of zymosan

Fig. 3 shows, for comparison, the effect of 10 mg/kg zymosan. Except for somewhat less hematological (Fig. 3C,D) and more expressed TXB_2 (Fig. 3F) changes, the effect of 10 mg/kg zymosan was essentially identical to that seen with 22 mg/kg AmBisome. Zymosan, being a well-known C activator, the practical identities of the measured physiological effects and C activation by AmBisome and zymosan provides strong support for the direct or indirect involvement of C activation in the observed hemodynamic and hematological changes.

3.3.3. Effects of Chol-PEG liposomes

In testing the effect of Chol-PEG liposomes on the same physiologic parameters as described above, we found that 60 mg/kg PL did not cause any change (not shown). Bolus injection of 300 mg PL/kg (Fig. 4) led to minor changes that resembled the effect of 22 mg PL/kg AmBisome (Fig. 1). We have seen relatively small (16%) but significant decrease in SAP (Fig. 4A), while HR did not change (Fig 4B). In parallel, leukopenia (but no leukocytosis) (Fig. 4C), thrombocytopenia (Fig. 4D), as well as a slight decrease in hemolytic activity (Fig. 4E) and a small rise in plasma TXB_2 could be observed (Fig. 4F). Thus, Chol-PEG liposomes

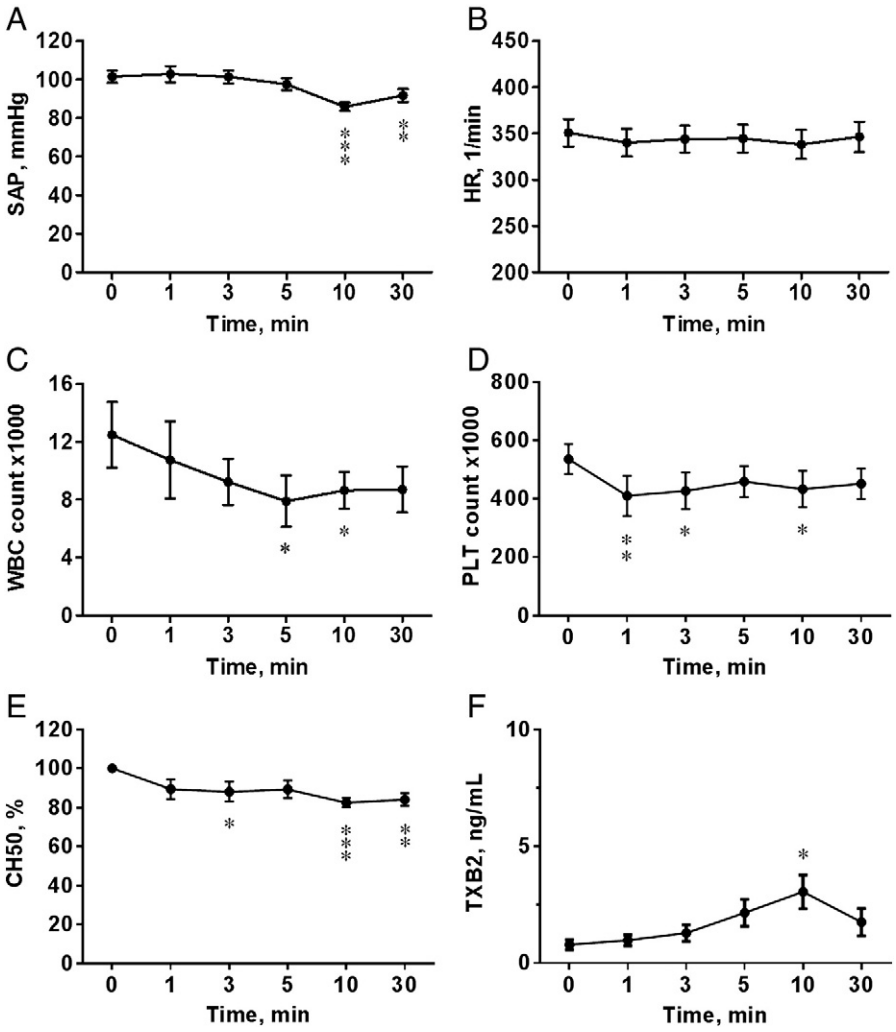

Fig. 4. Physiological changes in rats injected with Chol-PEG liposomes. Similar experiments as in Fig. 1, except that rats were injected with 300 mg/kg Chol-PEG (mean ± SE, n = 8).

were significantly less effective C activators in rats than AmBisome or zymosan, with signs of efficacy emerging only at 300 mg/kg dosing.

3.4. Physiological changes in pigs caused by AmBisome and Chol-PEG liposomes

Fig. 5A shows the cardiopulmonary effects of repetitive, bolus administration of 0.01 mg/kg AmBisome, leading to essentially identical, 3-fold (200%) rise of PAP with a 50% drop of SAP. This observation has two major implications. One is that pigs may be 3 orders of magnitude more sensitive to AmBisome reactions than rats (in Fig. 2A the dose of AmBisome that led to 40% drop of SAP in rats was 22 mg PL/kg, *i.e.*, 2200-fold higher). The second implication is that the reactogenic effect of AmBisome is not tachyphylactic, which is the opposite of what we have observed and reported for Doxil [17]. The third injection in Fig. 5A also shows that AmBisome did not tolerize the animal against Doxil reactogenicity, implying that this liposome does not induce cross-tolerance, either. The last zymosan injection showed that the reaction to AmBisome was very similar to that caused by a 50-fold higher dose of zymosan.

It should be pointed out that the tachyphylactic nature of the rats' reaction to AmBisome could not be established because of the toxicity of the large amount of lipid and volume that is needed for such experiments.

Fig. 5B shows an entirely different picture following repetitive administration of 10-times higher amount of Chol-PEG liposomes in a pig, which did lead to tachyphylaxis. Nevertheless, the tolerance could be broken by a 5-times higher dose of Chol-PEG vesicles (Fig. 5B).

Table 2 summarizes the results obtained in studies similar to those described above, wherein pigs (n = 3–5) were treated with repetitive boluses of AmBisome or Chol-PEG. In the case of AmBisome repeated dosing of 0.01 mg/kg did not induce tachyphylaxis. On the other hand, repeated boluses of 10-times more Chol-PEG liposomes (0.1 mg/kg) resulted in severely reduced peak responses, which implies tachyphylaxis. However, when the dose was further increased in the second or third injection (to 1 and 0.5 mg/kg), the tolerance disappeared.

4. Discussion

Hypersensitivity reactions to liposomal drugs, anticancer cytostatics solubilized with emulsifiers, antibody therapeutics, PEGylated proteins, intravenous iron formulations, radiocontrast media, enzymes and protein therapeutics seem to share numerous common features that are not characteristic of the classical IgE-mediated allergy. The World Allergy Organization call them “non-immune” allergy, or “non-immune” hypersensitivity, while other nomenclatures refer to pseudoallergy, anaphylactic, or anaphylactoid, or idiosyncratic reactions. The phenomenon was named CARPA 15 years ago, because of the purported causal role of C activation [1]. Since then the examples for CARPA being a major safety problem in pharmacotherapy using “vanguard” nanomedicines and biologics keep mounting, which underscores the urgent need to develop standardized *in vitro* and *in vivo* test systems to evaluate the risk,

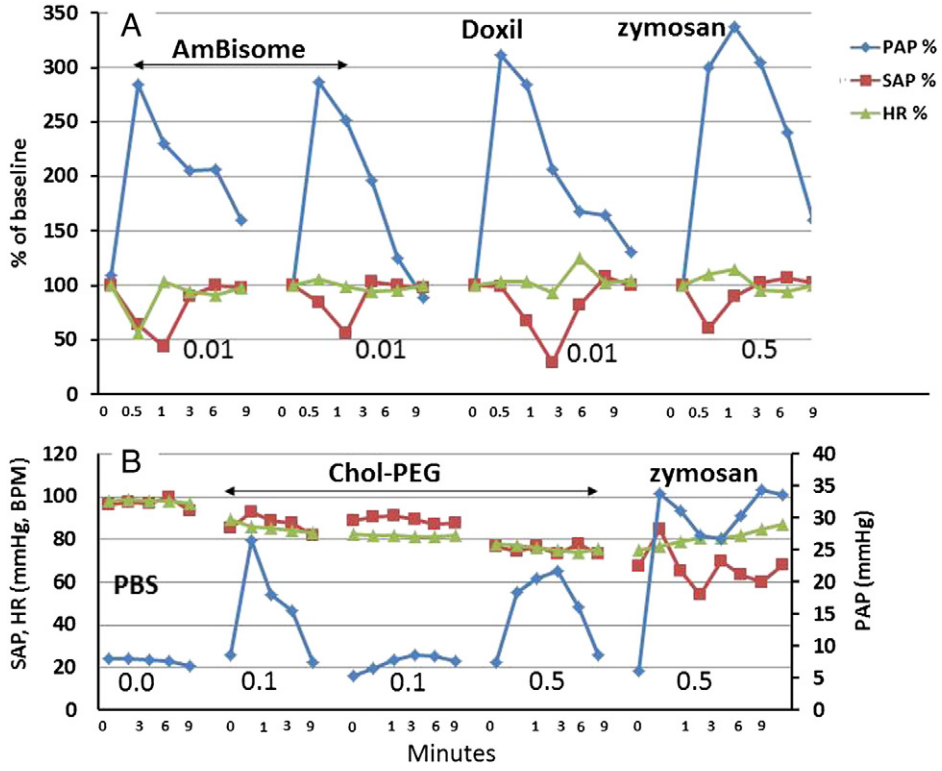

Fig. 5. Time course of cardiovascular (PAP, SAP and HR) changes caused in pigs by repetitive i.v. bolus injection of AmBisome and Chol-PEG liposomes. The administered liposomes and their doses (in mg PL/kg) are specified in the figures. The numbers under the X axes are minutes, when readings were taken.

and predict the rise of severe reactions. This study confirmed the previous evidence that pigs provide a highly sensitive model of CARPA [1,13–15,17], and also confirmed previous claims that rats are less sensitive for liposome-induced reactions than pigs. However, the present study went far beyond the observations on which the claim of low sensitivity was based [27,29], as it provided head-to-head comparison of the reactions in pigs and rats. The difference in sensitivity turned out to be in the range of 100 to 10,000, based on the dose–effect relationship information in this study. Obviously, a more accurate number would be preferable, but even these rough figures provide strong indication that the rat is not a sensitive model for immune toxicity screening or quantitative evaluation of the risk of CARPA. However, because the physiological changes in rats are essentially the same as those seen in pigs and man, rats still provide a good model for studying the mechanism of CARPA.

In addition to the difference in CARPA sensitivity, this study showed that the reactions to liposomes are of a very specific nature depending on the type of surface modification, be it charge (AmBisome), or polymer (Chol-PEG) and that this difference is consistent among distinct species. AmBisome, a highly charged nonPEGylated liposome with a phospholipid surface unmodified by PEG, which is widely used in the treatment of systemic fungal infections [30–36], and is known to

Table 2
Hemodynamic effects of repetitive bolus injections of AmBisome and Chol-PEG in pigs.

1st bolus			2nd bolus			3rd bolus		
Dose	Maximal change (%)		Dose	Maximal change (%)		Dose	Maximal change (%)	
mg/kg	PAP	SAP	mg/kg	PAP	SAP	mg/kg	PAP	SAP
				AmBisome				
	285	44		287	56		Not done	
	240	68	0.01	274	43			
0.01	177	114		167	104	0.05	363	73
	95	96		110	101		110	95
	126	103	0.05	126	97		Not done	
Mean	185	85		193	80		237	85
SD	78	28		83	29		179	16
				Chol-PEG				
	206	9		60	3		190	−2
0.1	392	−70	0.1	187	10	0.5	145	13
	46	8	1	175	25		0	0
Mean	215	−18	Mean	126	12		168	6
SD	239	219	SD	64	10		32	11

Entries are % changes relative to baseline, obtained in individual pigs by the equation: PAP or SAP_{max} / PAP or $SAP_{baseline}$ × 100) − 100, where PAP_{max}, $PAP_{baseline}$ and SAP_{max} and $SAP_{baseline}$ are mmHg PAP and SAP readings at the top of the hemodynamic changes following each injection, or at their baseline. Bolus injections were separated by 20–30 min recovery periods.

cause anaphylactic reactions in man [37], was stronger inducer of CARPA in both species than its identically sized (~100 nm), uncharged PEGylated counterpart, Chol-PEG liposomes. PEGylated liposomes are widely used in the treatment of cancer (*e.g.* Doxil/Caelyx, which is PEGylated liposomal doxorubicin). However, these liposomes have a slight negative charge as a result of the anionic PEGylated phospholipid conjugate that is used. To single out the effect rendered by PEGylation, we used PEG conjugated to Chol instead, which is uncharged, and found in a previous study that these liposomes also cause major C activation [28]. The finding that the two liposomes caused major C activation in human serum while their reactogenic dose significantly differed from each other in these animals shows, at least for this special case, the lack of absolute correlation between *in vivo* reactogenicity of liposomes and their C activating effect in human serum *in vitro*.

The reason for the substantial difference in CARPA sensitivity between the pig and the rat has not been clarified to date. The phenomenon can most easily be rationalized by the presence of high-secretory pulmonary intravascular macrophages (PIM cells) in the microcirculation of pig lungs [38–40], making the lung as primary CARPA responder organ [15]. With the cardiopulmonary circulation temporarily blocked by massive pulmonary and coronary vasoconstriction and leukothrombotic microembolism, the rapid progression into cardiac anaphylaxis and shock can be explained [41]. In view of the lack of such cells in the lung of rats, the most likely primary responder organ is the liver, where the Kupffer cells take the role of PIM cells, however, the entailing physiological changes are much less severe. Studies are underway in our laboratories to explore the involvement of Kupffer cells and liver circulatory changes in the CARPA phenomenon in rats.

Finally, the strong C activating effect of Chol-PEG liposomes *in vitro*, and their strong reactogenicity (at least in pigs) need to be commented, as these effects are unexpected and are yet unexplained. Namely, the *in vitro* studies in NHS showing that the neutral (Chol-anchored) liposomes caused strong C activation contradict the proposal that it is the anionic phosphate group on PEG-PE that is responsible for C activation by PEGylated liposomes [5]. Our data suggests that C activation by PEGylated nanoparticles may be more complex and critically depend on surface properties other than charge, thus the original intention to eliminate C activation by using neutral anchor for 2K-PEG does not necessarily solve the CARPA problem.

As for the mechanism of C activation by Chol-PEG liposomes, we showed previously [28] that it proceeds *via* the alternative pathway, ruling out a role for natural antibodies (*e.g.*, anti-cholesterol, anti-PEG and anti-phospholipid antibodies). The enigma has, however, not yet been solved, and one may speculate that Chol is unlikely to hold the PEG firmly to the liposomes after being exposed to the serum or blood, but it rather detaches from the bilayer and enters in complex interactions with plasma elements. This needs to be explored in future studies, however, it should be stressed that increased C activation by PEGylated neutral liposomes *via* the alternative pathway, entailing substantial anaphylactic potential in pigs, is an unprecedented phenomenon in experimental immunology and toxicology that could be utilized in unveiling the mechanism of C activation and CARPA.

In summary, this study provided evidence for a large difference between pigs and rats in sensitivity for CARPA, and for differential impact of vesicle charge and PEG graft on CARPA in these species with or without tachyphylaxis. This information helps developing animal protocols for safety testing of i.v. drugs with regard to CARPA.

Acknowledgments

The financial support from the following grantors is gratefully acknowledged: National Development Agency of Hungary (TECH-08-D1-2008-0030 “Nanomedi”); Miskolc University, TÁMOP-4.2.1.B to “Applied Materials and Nanotechnology Center of Excelence”; EU project FP7-NMP-2012-LARGE-6-309820 (NanoAthero); Phospholipid Research Center, Heidelberg, Germany, Grant to Dr Metselaar; and Gedeon Richter NyRT, support to the Nanomedicine Research and Education Center at Semmelweis University.

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