Prevention of infusion reactions to PEGylated liposomal doxorubicin via tachyphylaxis induction by placebo vesicles: A porcine model

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ABSTRACT

PEGylated liposomal doxorubicin (Doxil) has been used in cancer chemotherapy for 16 years. Clinical experience shows that it can cause mild-to-severe hypersensitivity (infusion) reactions, which are manifestations of complement (C) activation-related pseudoallergy (CARPA). Although in most cases CARPA is inconsequential, a main symptom, cardiopulmonary distress, may be life threatening in hypersensitive individuals. To date, the prevention of Doxil-induced CARPA is based on premedication and a slow infusion protocol. The present study suggests desensitization by Doxil-like empty liposomes, called placebo Doxil (Doxebo), as an alternative strategy, which is based on the tachyphylactic nature of Doxil reactions. Doxebo-induced tolerance to Doxil was shown to develop within minutes and to be specific to Doxil-like PEGylated liposomes. The procedure of desensitization involves slow, low-dose pre-infusion of Doxebo before Doxil treatment which minimizes the ensuing physiological changes or keeps them subclinical. Although the mechanism of tolerance induction is not yet clear, the effector arm of C response is unlikely to be affected, as the vascular reactivity of desensitized pigs to zymosan remains intact. Desensitization with empty vesicles represents a novel approach for reducing the risk of anaphylactic reactions to drug carrier liposomes. The underlying immediate, most likely passive silencing of an innate immune response may represent a novel mechanism of tolerance induction which may work for other reactogenic nanosystems as well.

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1. Introduction

Liposomal doxorubicin (Doxil), also referred to as PEGylated liposomal doxorubicin (PLD), is the first “nano-drug” approved by the FDA [1]. It has been used for the treatment of cancer since 1995 [2,3], when it received accelerated approval for the treatment of chemotherapy-refractory AIDS-related Kaposi’s sarcoma. Since then, the ~400 completed or ongoing clinical trials testing the use of Doxil as a single drug or in combination with other agents in various cancers [4] hold promise for its expanded use in the future. The advantages of Doxil over free doxorubicin include the reduction of cardiac toxicity and extended duration of anticancer action; however, these benefits are not without a price, as Doxil has its own specific adverse effects, such as the hand–foot syndrome [5] and the increased risk for hypersensitivity reactions (HSRs), also known as infusion reactions [5–10]. The latter, non-IgE-mediated (pseudoallergic) reactions were previously shown to arise, at least in part, from the capability of Doxil to induce complement (C) activation [10–13].

Although in most cases Doxil-induced C activation-related pseudoallergy (CARPA) [14–17] is mild and inconsequential, the cardiopulmonary distress it entails may be life threatening in a few hypersensitive individuals [5]. The exploration of possible methods for avoiding Doxil-induced CARPA represents an important goal for improving the safety and therefore the clinical use of this drug, as well as that of other similarly reactogenic “nanodrugs.” The particular goal of this study was to present a “proof of concept” for the possible utility of a desensitization approach against Doxil-induced CARPA, using placebo Doxil, which we have named “Doxebo.”
2. Materials and methods

2.1. Materials

Commercial Doxil was obtained from the pharmacy of Semmelweis University (in this paper we refer to Caelyx as Doxil, as the two names represent the same drug). It contains doxorubicin, 2 mg/ml (4.22 mM); fully hydrogenated soy phosphatidylcholine (HSPC), 9.58 mg/ml; cholesterol (Chol), 3.19 mg/ml; 2K-PEG-DSPE (N-carboxymethyl-poly(ethylene glycol methyl ether)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine triethyl ammonium salt with a polyethylene glycol (PEG) moiety of average molecular mass of 2000 Da, 3.19 mg/ml; ammonium sulfate, ~0.2 mg/ml; histidine (10 mM, pH 6.5); and sucrose (10%). Total phospholipid: 12.8 mg/ml (13.3 mM). Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG) and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Doxorubicin was from TEVA Pharmaceuticals (Peachtree Tikva, Israel).

2.2. Preparation and characterization of placebo Doxil (Doxeo)

The freeze-dried lipid components of Doxil (listed above) were hydrated in 10 ml sterile pyrogen-free normal saline (NS) by vortexing for 2–3 min at 70 °C to form multilamellar vesicles (MLVs). The MLVs were downsized through 0.4- and 0.1-μm polycarbonate filters in two steps, 10 times through each, using a 10-ml extruder barrel from Northern Lipids (Vancouver, British Columbia, Canada) at 62 °C. Liposomes were suspended in 0.15 M NaCl/10 mM histidine buffer (pH 6.5). The size distribution and phospholipid concentration of DoxoE were characterized as described earlier [18].

2.3. Determination of bacterial endotoxin (LPS) in liposomes

The LPS content of liposomes prepared for this study was determined by a limulus amebocyte lysate assay (PYROGENT Plus, Cambrex Bio Science, East Rutherford, NJ), after dissolving (96% ethanol) and separating (ultrafiltration using a 20-kDa cut-off membrane) the lipids from LPS [19]. Acceptance criteria as pyrogen-free were ≤ 0.5 EU/ml (0.01–0.25 ng LPS/ml).

2.4. Animal studies

Experiments using pigs were performed at Semmelweis Medical University in Hungary and at the Uniformed Services University of the Health Sciences (USUHS). They were approved by the local Animal Subject Review Committees, and we followed their guidelines, treating the animals humanely. Swine (25–40 kg) of both sexes were purchased from approved local vendors. They were sedated with i.m. ketamine (500 mg) and anesthetized with 2% isoflurane or with i.v. xylazine/ketamine mixture and Nembutal (pentobarbital, 30 mg/kg), via the ear vein. A fluid (Salsol A or Ringer) supply maintaining circulatory stability was provided via the left external jugular vein. Ventilation (upon isoflurane anesthesia) was maintained using the anesthesia machine or was assisted by a Harvard Ventilator (Harvard Apparatus, Cambridge, MA).

Surgery was performed to cannulate the right external jugular vein for drug injections and placing the Swan-Ganz catheter in the pulmonary artery to measure pulmonary arterial pressure (PAP). The right femoral artery was also cannulated for blood sampling and to measure systemic arterial pressure (SAP). The ECG was traced at the standard Einthoven’s 3-lead detection points. Hemodynamic parameters (PAP, SAP), heart rate, and ECG were continuously monitored using, among others, the “S.P.E.L. Advanced Hemosys” data acquisition system (Experimetria Ltd, Budapest, Hungary). Other details of surgery, instrumentation, and hemodynamic analysis were described previously [13,14,20–22]. The indicated amounts of liposomes and other test materials were diluted in PBS and injected into the pulmonary artery as boluses, via the pulmonary arterial catheter. Liposomes were flushed into the circulation with 5 ml of PBS or Ringer solution.

2.5. Quantifying of physiological changes during liposome reactions

Liposome reactions were quantified with the cardiac abnormality score (CAS), a semi-quantitative measure of the severity of cardiac electric, circulatory (systemic and pulmonary), and skin changes during CARPA [21]. Scores of 0–5 imply, respectively, no response (CAS: 0), minimal (CAS: 1), mild (CAS: 2), moderate (CAS: 3), severe (CAS: 4), and lethal (CAS: 5) reactions [21].

2.6. Statistical analysis

The significance of PAP and SAP changes caused by Doxil was computed by the Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%, or by unpaired t test (two tailed), as indicated. Differences between groups were considered significant at \( P<0.05 \).

3. Results

3.1. Tachyphylactic blood pressure changes caused by repetitive injections of Doxil

As detailed in previous studies, the cardiovascular changes caused by a single bolus of Doxil in pigs included a major rise of pulmonary arterial pressure (PAP) and rise or drop of systemic arterial pressure (SAP) with or without significant changes in heart rate and cardiac rhythm [13,14,20–22]. However, we have not shown previously that these changes ensued only after the first liposome bolus. Subsequent, repeated injections of the same or higher doses of Doxil in the same animal caused entirely different hemodynamic and cardiac electric changes: the reactions decreased or entirely disappeared after the second or third bolus, implying self-induced tolerance, also known as tachyphylaxis. The phenomenon is illustrated in Fig. 1 by the real-time PAP curves recorded in a pig repeatedly injected with Doxil. It is seen that the first bolus led to a dramatic >400% rise in PAP, and it took >20 min until the baseline returned to normal. After that, the second, identical bolus caused only a minor, biologically irrelevant ~20% rise, while a third, five-fold higher dose of Doxil caused no visible change in PAP, implying full tolerance. In contrast, while being tolerant to Doxil, the C-activator zymosan caused pulmonary hemodynamic changes almost identical to those seen at the first Doxil bolus. Thus, the tachyphylactic response to Doxil was not due to general vascular toxicity or depletion of the chemical mediator(s) of CARPA in the efferent arm of cardiovascular response. The rapid (minutes) manifestation of tolerance, taken together with the fact that the development of “classical” immune memory usually takes days to weeks, suggests that the underlying down-regulation of immune response may be passive.

Table 1 summarizes the PAP changes after the first and second bolus injections of Doxil in several (8–12) animals. As in Fig. 1, the data indicate significant elevation of PAP relative to baseline after both treatments; however, the rise of PAP after the second injection (from a mean of 17–19 mmHg) is biologically irrelevant in contrast to the rise (from a mean of 17–41 mmHg) after the first injection, which represents the biological limit of pulmonary hypertension. Table 2 confirms the major difference between the first and subsequent reactions to Doxil for the cardiac abnormality score (CAS) as
well, which is a comprehensive measure of all clinical changes during CARPA (see Materials and methods). These data together provide evidence for the development of generalized tolerance against CARPA that is not limited to the pulmonary response.

3.2. Changes of systemic arterial pressure during Doxil-induced CARPA

Unlike the consistent major rise with subsequent diminution of PAP following repetitive injections of Doxil (Fig. 1 and Table 1), the parallel changes in SAP showed substantial variation in different pigs. This observation is illustrated in Fig. 2, which shows individual SAP values for 5-6 animals at maximal deviation from baseline (SAP_{max}). It is seen that the first Doxil bolus led to either a rise, or fall, or no change in SAP_{max}, resulting in a small average change relative to baseline, with high variation (SD of SAP_{max} = 44). In keeping with the generalized tachyphylactic effect of first bolus, the 2nd and 3rd injections of Doxil with the same, and then with a 5-fold larger dose, led to minimal changes with significantly smaller variation (SD = 7, 8). Finally, two repeat boluses of zymosan led to very similar changes as the first Doxil bolus and a subsequent zymosan injection also resembled first-dose reactions; thus, the reaction to AmBisome was non-tachyphylactic and non-cross-tolerogenic.

3.3. Variations of liposome-induced tachyphylaxis

The tachyphylactic nature of pseudoallergic reactivity of Doxil is in sharp contrast with the cardiovascular effects of large multilamellar liposomes (LMV) composed of DMPC/DMPG/Chol (45:5:50 mole ratios), which were shown to cause identical reactions in pigs over a long time (8 injections over 8 h [14]). Thus, tachyphylaxis may not be an inherent property of liposomal CARPA. To further explore possible variations of tachyphylaxis following repetitive injections of different liposomes and other C activators, we subjected pigs to serial injections of liposomal amphotericin B (AmBisome), Doxil, and zymosan, in different combinations. As illustrated with a typical experiment (Fig. 3), AmBisome triggered a reaction similar to that caused by Doxil; however, the reaction to a second dose was not attenuated, indicating a lack of tachyphylaxis. In addition, a subsequent Doxil bolus and a final zymosan injection also resembled first-dose reactions; thus, the reaction to AmBisome was non-tachyphylactic and non-cross-tolerogenic.

Fig. 4 shows yet another variation of tachyphylactic CARPA: non-gradual, partial tachyphylaxis observed in the systemic hypotensive effect of repetitively injected zymosan. In this case, after 2 identical

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**Table 1**

<table>
<thead>
<tr>
<th>Pulmonary blood pressure responses of pigs to repeated bolus injections of the same (0.06 mg phospholipid/kg) dose of Doxil.</th>
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<tr>
<td>Order of injections</td>
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<tr>
<td>Mean PAP baseline, mm Hg</td>
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<td>SD</td>
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<td>P, baseline vs. PAP_{max}</td>
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P_{1st} vs. 2nd injection**

P_{PAP, max} at the peak of hypertensive response; * P value determined by the Wilcoxon matched-pairs signed rank test (confidence interval 95%); and ** P value determined by the unpaired t test (two tailed). Significant changes are italicized.
CARPA in pigs, compared to Doxil [13]. These considerations and facts taken together led us to test the feasibility of tolerizing pigs to Doxil with Doxebo.

3.4. Utilization of tachyphylaxis for desensitization to Doxil: A concept and its proof

The tachyphylactic nature of hemodynamic response to Doxil raises the possibility of using the effect for the prevention of HSRs to Doxil, by giving the drug as second injection after a first tolerizing dose whose hemodynamic reactivity is reduced or eliminated. Along this line, we reported previously that slow infusion versus bolus injection of liposomes significantly reduced CARPA in pigs [20], and also, that, like Doxil, doxorubicin-free (empty) liposomes (Doxebo) caused milder C activation in human serum, as well as milder CARPA in pigs, compared to Doxil [13]. These considerations and facts taken together led us to test the feasibility of tolerizing pigs to Doxil with Doxebo.

As shown in Fig. 5, bolus injection of Doxebo (0.01 mg phospholipid/kg) almost fully prevented the major (300–400% rise of PAP, see Fig. 1 and Table 1) reaction caused by Doxil as first injection. The figure also shows that, like Doxil, Doxebo did not prevent the reaction caused by zymosan, thus its protective effect is specific for Doxil (at least versus zymosan). Nevertheless, Doxebo caused a relatively small (50%), but significant pulmonary reaction, suggesting that bolus injection of Doxebo still carries a risk for clinically manifested HSR.

The data summarized in Fig. 6 suggest that this problem might be solved by slowing the administration of Doxebo: the Doxil-induced rise of PAP in 5 pigs was reduced to below 20% (a biologically irrelevant change), by way of slow infusion (over 15–20 min) of Doxebo, applied shortly (within 15–20 min) before Doxil injection. Because the reaction caused by zymosan was not affected, the tolerizing effect of Doxebo seems to be similar to that of Doxil in terms of specificity. Importantly, the reactogenicty of Doxebo also remained below about 20%, raising the possibility that the above approach might provide significant protection against Doxil reactions with significantly minimized risk for clinical reaction to Doxebo itself.

4. Discussion

4.1. Current approaches to the prevention of infusion hypersensitivity

Today the prevention of HSRs to Doxil and many other infusion therapies, including micellar drugs and monoclonal antibodies, is based on premedication with known anti-inflammatory and anti-allergic agents (dexamethasone, ibuprofen, acetaminophen, antihistamines). In addition, it is standard practice to infuse reactogenic medicines slowly, in particular, at the start of infusion. If signs of a reaction appear, interruption or abandonment of the infusion is necessary [3,23–26]. Although highly effective, the substantial (~10) percentage of HSRs to Doxil and other nanomedicines and antibody therapeutics [5,25,27] highlight the fact that the available preventive measures do not provide full security against infusion hypersensitivity. There is a clear need to find alternative ways to avoid these reactions, particularly in the case of therapies where iatrogenic triggering of cardiopulmonary distress may be disastrous, e.g., in patients with cardiovascular diseases and those proneness allergy (atopy).

4.2. Types of tachyphylactic cardiopulmonary reactions to complement activators in pigs and their novelty

Our previous and present experiments on porcine CARPA suggest the existence of at least 3 types of cardiopulmonary responses to particulate substances that activate C: (1) non-tachyphylactic response, exemplified by zymosan, which is characterized by a 300–400% rise of PAP, lasting for 10 min (Fig. 2); (2) tachyphylactic response, exemplified by slow infusion of doxorubicin-free liposomes: the reaction caused by zymosan was not affected, the tolerizing effect of Doxebo seems to be similar to that of Doxil in terms of specificity, raising the possibility that the above approach might provide significant protection against Doxil reactions with significantly minimized risk for clinical reaction to Doxebo itself.

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by the reactions caused by AmBisome in the present study and by MLV in an earlier study [14]; (2) partial tachyphylaxis, shown in the present study (for the SAP changes) caused by zymosan and in an earlier study (for the PAP changes) caused by large (25 kDa) polyethylenimine (PEI) nanoparticles with or without PEGylation [28]; and (3) full tachyphylaxis, induced by Doxil and Doxebo in the present study.

Tachyphylaxis, in general, is a term for self-desensitization, i.e., a self-induced decrease of a (patho)physiological response to a drug or agent as a consequence of prior exposure. Textbook examples of tachyphylactic drug effects include the anagelsic and hallucinogenic effects of opioids, LSD and psilocybin, or the tolerance to ephedrine, calcitonin, nitroglycerine, nicotine, hydralazine, dobutamine, and desmopressin. However, unlike the tachyphylaxis shown in the present study, the decrease of response in the above examples takes place slowly, over days or weeks. To our knowledge, the immediate and full tachyphylaxis described here for Doxil and Doxebo is not a commonly recognized phenomenon in immunology, physiology, or pharmacotherapy.

4.3. Possible mechanisms of tolerance induction

The rapidity of tachyphylaxis (within minutes) argues against development of immune memory via specific cell (lymphocyte) activation. Because zymosan displays full reactivity in animals tolerized by Doxil, massive C consumption, or depletion of a component in the effector arm of CARPA can also be ruled out. Although at this time there is no experimental evidence, the phenomenon can most easily be rationalized either by consumption of an early mediator of CARPA, such as natural anti-PEG antibodies [29], or by down-regulation of a signaling process in cells that mediate the reaction. Whether the signal silencing involves anaphylatoxin receptors, or perhaps pattern recognition receptors (PRRs) that sense the PEG-DSPE on Doxil as a pathogen-associated molecular pattern (PAMP) [30], remains to be investigated.

Regarding the role of PEG, and, hence, the stealth character of Doxil, it should be noted that large PEI polymers induced similar tachyphylactic reactions in pigs as Doxil, which effect was variably modulated by PEGylation of PEI [28]. However, the pig reactions to these polymers did not correlate with their C activation (at least in human serum), suggesting that cells mediating the HSR in pigs were directly triggered by polymeric or polymer coated particles, possibly via their PRRs. That PEG on the surface of liposomes (i.e., stealthness) may in fact be a critical factor in tachyphylaxis is also supported by the finding in the present study that PEG-free AmBisome, which is similar to Doxil in size, and which causes similar first reactions in pigs as Doxil, does not lose reactogenicity upon repeated injections (i.e., its effect is non-tachyphylactic). Finally, the data available raise the possibility that the “array” nature of the PEG corona on liposomes becomes immune active only on a (negatively) charged membrane platform. This proposal is suggested by earlier results showing significant reduction of the C activating capability and in vivo reactogenicity of PEGylated liposomes when the polymer was grafted onto the vesicles via neutral phospholipids (DS or DSG) [13], or when the phosphate oxygen moiety of the PEG-anchor phospholipid (DPPE) was methylated [31].

4.4. The “double-hit” and “PIM” theories on porcine CARPA

The above discussed facts and considerations of the mechanism of Doxil-induced tachyphylaxis raise the possibility that Doxil reactions arise as a consequence of a double hit on CARPA-mediating cells, i.e., concurrent stimulation of these cells by anaphylatoxins and by physical binding (or uptake) of liposomes. When one (or both) hit(s) weaken(s), which occurs for some reason upon repetitive injections, tachyphylaxis develops. As for the localization and identity of CARPA-mediating cells in pigs, the rapidity and predominance of pulmonary reaction points to a key role of pulmonary intravascular macrophages (PIM cells), which are present in large amounts in pigs [32,33] and which, just like other macrophages in vitro [34], are probably able to take up liposomes on a time scale of minutes.

4.5. Clinical advantages of Doxebo use and broader implications of tachyphylaxis induction with empty liposomes

The method of anaphylaxis prevention by tolerance induction with “empty” nanocarrier placebo has unique advantages for the case of Doxil, and has broader implications as well for future developments of reactogenic and immunogenic nanomedicines. In particular, our CARPA data from the porcine model suggest that a short (15–30 min) infusion of Doxebo might significantly reduce, or entirely prevent the HSR to subsequently administered Doxil. In pigs this protective effect may be maintained for at least 24 h post Doxebo administration (data not shown). The method might save patients from severe, and in rare cases potentially lethal, adverse immune reactions. Pretreatment with Doxebo, if proven to have clinical utility, would also lead to shorter infusion protocols and reduced spending on preventative interventions and first-aid emergency measures. The proposed approach of tolerance induction might also be utilized for other nanomedicine-induced hypersensitivity reactions, provided the CARPA-genic effects of these nanomedicines are tachyphylactic.

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