Animal Models of Complement-Mediated Hypersensitivity Reactions to Liposomes and Other Lipid-Based Nanoparticles

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Intravenous injection of some liposomal drugs, diagnostic agents, micelles and other lipid-based nanoparticles can cause acute hypersensitivity reactions (HSRs) in a high percentage (up to 45%) of patients, with hemodynamic, respiratory and cutaneous manifestations. The phenomenon can be explained with activation of the complement (C) system on the surface of lipid particles, leading to anaphylatoxin (C5a and C3a) liberation and subsequent release reactions of mast cells, basophils and possibly other inflammatory cells in blood. These reactions can be reproduced and studied in pigs, dogs and rats, animal models which differ from each other in sensitivity and spectrum of symptoms. In the most sensitive pig model, a few miligrams of liposome (phospholipid)
can cause anaphylactoid shock, characterized by pulmonary hypertension, systemic hypotension, decreased cardiac output and major cardiac arrhythmias. Pigs also display cutaneous symptoms, such as flushing and rash. The sensitivity of dogs to hemodynamic changes is close to that of pigs, but unlike pigs, dogs also react to micellar lipids (such as Cremophor EL) and their response includes pronounced blood cell and vegetative neural changes (e.g., leukopenia followed by leukocytosis, thrombocytopenia, fluid excretions). Rats are relatively insensitive inasmuch as hypotension, their most prominent response to liposomes, is induced only by one or two orders of magnitude higher phospholipid doses (based on body weight) compared to the reactogenic dose in pigs and dogs. It is suggested that the porcine and dog models are applicable for measuring and predicting the (pseudo)allergic activity of particulate “nanodrugs”.

Keywords  nanomedicines, liposomes, adverse drug reactions, complement, animal models, hypersensitivity

Introduction

One of the challenges of “nanomedicine” today is a need for being alert to potential hypersensitivity reactions (HSRs) to i.v. injected particulate substance, such as liposomes, micelles and other natural or synthetic particles in the submicron size range. The multifactorial etiology, variability and unpredictability of these reactions represent a major problem in studying their mechanism and prevention in humans, making the animal models discussed in this review particularly valuable.

Research over the past few years provided substantial evidence that many of these HSRs, also referred to as “infusion” or “idiosyncratic” reactions, are due to complement (C) activation by the lipid particle. Hence, the phenomenon was dubbed “C activation-related pseudoallergy” (CARPA) (Szebeni, 2001, 2004, 2005), a novel subclass of “nonallergic drug hypersensitivity”, the new term of non-IgE-mediated HSRs adopted by the World Allergy Organization (Johansson et al., 2004).

The clinical picture of CARPA includes cardiovascular (tachy- and bradycardia arrhythmia, hypo- and hypertension, chest pain, back pain) respiratory (tachypnea, bronchospasm, dyspnoea) and cutaneous (flushing, urticaria, erythema, pruritus) symptoms. Unlike classical, IgE-mediated HSRs, CARPA arises at the first treatment (no prior exposure to allergen), and the reactions are usually less severe or are absent upon repeated exposure. Also, the frequency of CARPA in the 5–45% range (see later) is much higher than classical anaphylactic reactions to drugs (for example, penicillin allergy occurs in <2%).

While transient, mild reactions are viewed as an inconvenience greatly outweighed by the benefits of treatment, severe reactions can cause major anxieties, disruptions and extra expenses, most importantly, exclusion of the patient from receiving a potentially useful, life-saving treatment. These reactions can also be fatal in a small percentage of hypersensitive individuals, mainly those with a history of severe allergy and/or heart disease (Szebeni, 2005; Szebeni et al., 2006).

Animal Models of CARPA

Although it is likely that almost all animals with humoral immunity (wherein complement proteins are ubiquitously present) respond in some way to drug exposure mimicking microbial invasion, we focused here only on the responses in pigs, dogs and rats. Tables 1 and 2 specify the spectrum of symptoms and particle (phospholipid) dose dependence of CARPA in these species, respectively.
Species Dependence of Symptoms

As shown in Table 1, some of the physiological and laboratory changes during liposome-induced CARPA are identical in all three animal species and man, while others substantially differ. Hyper- or hypotension are universal but inconsistent symptom; depending on reaction strength and numerous yet undefined conditions, they may alternate or one or the other predominate. For example, in pigs, mild reactions usually entail hypertension while...
strong reactions trigger hypotension (Szebeni et al., 2006). Pulmonary hypertension probably represents a constant manifestation of CARPA; however, because of the invasive, surgical skill requiring technique of its measurement (Swan-Ganz catheterization; Szebeni et al., 2003), it is mainly in pigs that this parameter has been systematically followed during liposome reactions (Szebeni et al., 1999, 2000, 2006). Cutaneous changes (flushing and rash) represent a universal, very frequent symptom in man and pigs (~40–50% of pigs display it in the case of strong liposome reactions), while they occur only occasionally in dogs and never in rats (unpublished observations). Dogs display, in addition to the hemodynamic changes and occasional skin reaction, profound, but reversible blood cell changes (leukopenia with or without leukocytosis, thrombocytopenia (Henricsson and Bergentz, 1978) as well as vegetative neurological imbalance manifested in intense salivation, urination and (occasionally) defecation. In pigs, the most likely sources of TXA₂ production (the secondary mediator causally involved in porcine CARPA (Szebeni et al., 1999), are pulmonary intravascular macrophages (PIM cells; Bertram et al., 1988; Gaca et al., 2003; Ostensen et al., 1992), which are directly exposed to the circulation (Chitko-McKown et al., 1991). Taken together, it is the pig whose symptoms most resemble to those of humans, inasmuch as the cutaneous changes are clearly identical, while the hemodynamic and respiratory alterations measured in pigs are likely to be present to some degree in all reactive humans as well, based on the well known manifestations of HSR (dyspnea, chest pain, back pain, light-headedness, transient confusion).

Regarding the variability of the clinical picture of CARPA, it is important to point out that the spectrum of symptoms critically depends on the severity of reactions. This is illustrated in Fig. 1, wherein the ECG alterations observed in two pigs, experiencing HSR from different doses of Doxil, are entirely different: in one pig the reaction was associated with severe but reversible bradyarrhythmia (panel A), while the other developed tachycardia with ventricular fibrillation which would have been lethal if the animal had not been resuscitated by i.v. epinephrine (panel B). Because of this substantial variation of symptoms, in

![Figure 1](https://example.com/figure1.png)

**Figure 1.** ECG changes in pigs treated with increasing doses of Doxil: 0.2 mg lipid/kg (A) and 0.5 mg lipid/kg (B) mg lipid/kg. The figure illustrates the variability of ECG alterations depending on dose, and, hence, reaction severity. Modified from Szebeni et al. (2006). SAP, systemic arterial pressure.
an effort to quantify the pigs’ cardiopulmonary reaction to liposomes, we introduced an arbitrary but quantitative scoring system that took into consideration all hemodynamic and ECG changes, and differentiated between groups of symptoms with quantitatively distinguishable levels of reaction severity (Szebeni et al., 2006). Table 3 shows this classification, based on the analysis of 111 liposome reactions. We differentiated five categories with increasing “cardiac abnormality scores” (CAS) in the 1–5 range, reflecting increasing severity from mild to lethal. The system allowed us to quantitatively correlate HSRs with C activation in blood, and propose that liposome-induced CARPA in pigs provides a unique large animal model of human cardiac anaphylaxis (Szebeni et al., 2006).

### Table 3

<table>
<thead>
<tr>
<th>ECG abnormalities</th>
<th>Associated hemodynamic and cardiorespiratory alterations</th>
<th>Qualitative description</th>
<th>CAS(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrhythmia episodes, transient tachycardia</td>
<td>No or minimal changes in PA, SAP, CO, PAP, and pCO(_2)</td>
<td>Minimal</td>
<td>1</td>
</tr>
<tr>
<td>Longer lasting arrhythmia, with tachycardia</td>
<td>Moderate rise of SAP and reduction of PA, no or minimal changes in CO, PAP, and pCO(_2)</td>
<td>Mild</td>
<td>2</td>
</tr>
<tr>
<td>Major arrhythmias with tachycardia and/or bradycardia</td>
<td>Initial rise followed by moderate declines in SAP, PA, CO, and pCO(_2), moderate rise of PAP</td>
<td>Moderate</td>
<td>3</td>
</tr>
<tr>
<td>Major arrhythmias with tachycardia and/or bradycardia, ST depression/T wave changes</td>
<td>Dramatic and extended declines in SAP, PA, CO, and pCO(_2), major rise of PAP</td>
<td>Severe</td>
<td>4</td>
</tr>
<tr>
<td>Major arrhythmias with tachycardia and/or bradycardia, ST depression/T wave changes, cardiac arrest with or without ventricular fibrillation</td>
<td>Dramatic, extended and irreversible declines in SAP, PA, CO, and pCO(_2), maximal rise of PAP. Fatal without CPR(^2)</td>
<td>Lethal</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^{1}\)CAS, cardiac abnormality score, an arbitrary rank based on the complexity of ECG abnormalities (column 1) and number and severity of hemodynamic and respiratory abnormalities listed in column 2. \(^{2}\)Cardiopulmonary resuscitation with chest compressions, epinephrine and/or electroversion. Further abbreviations: PA, pulse amplitude; SAP, systemic arterial pressure; PAP, pulmonary arterial pressure; CO, cardiac output, pCO\(_2\), expiratory (end-tidal) CO\(_2\). (from Szebeni et al., 2006).

Dose Dependence of CARPA in Man and Different Animals

In light of the phospholipid dose range causing significant cardiorespiratory and cutaneous symptoms in man, pigs and dogs (Table 2), the dose dependence of liposome reactions is near equal in these species. Remarkably, miligram amounts of phospholipids, for example 100–200 microliters from undiluted Doxil, can cause life-threatening reactions in pigs.
and dogs, just as in man. Rats, on the other hand, are two-to-three orders of magnitude less sensitive to liposomes (Table 2), at least to those containing <50% cholesterol (Baranyi et al., 2003).

The resources and calculations of the above dose ranges were as follows. The human reactogenic dose range was obtained from the study of Chanen-Khan et al (Chanen-Khan et al., 2003) wherein eight clinical studies were compared (de Marie, 1996; Gabizon et al., 1994; Gabizon and Muggia, 1998; Gordon et al., 2000; Hubert et al., 2000; Koukourakis et al., 1999; Lyass et al., 2000; Muggia et al., 1997; Northfelt et al., 1997; Uziely et al., 1995) to estimate the reactogenic dose range of Doxil (Chanen-Khan et al., 2003). The analysis showed 20–80 mg/m², which (calculating with 1.6–2 m² average body surface for a 60–80 kg human), implies ~0.25–2 mg/kg (doxorubicin) total dose. Considering that the phospholipid to doxorubicin weight ratio in Doxil is ~7:1, and that during the first minutes of infusion (at 1/5th of the final infusion rate, which can already trigger a reaction) 1/100 to 1/500 of the total amount of drug reaches the circulation of patients, the minimum reactogenic dose of phospholipids, in the form of reactogenic liposomes, is roughly 0.01–0.2 mg/kg in man (Table 2).

In pigs, studies from our laboratories have established 2–10 mg liposome lipid boluses from DMPC/DMPG/Chol (50:5:45 mole ratio) multilamellar vesicles (MLV) as reactogenic dose range, causing significant cardiopulmonary changes (Szebeni et al., 1999, 2000). Five mg total lipid (~3.6 mg phospholipid), corresponding to the ED₅₀, caused major, yet reversible pulmonary hypertension that was quantitatively repeated several times (Szebeni et al., 1999, 2000). The corresponding 0.01–0.3 mg phospholipid/kg therefore closely overlaps with the human reactogenic dose. In other pig studies using Doxil as reaction trigger, boluses containing 0.03–0.3 mg phospholipid/kg caused significant cardiopulmonary reactions (Szebeni et al., 2000, 2006).

It should be noted with regard to the porcine model of CARPA that while all pigs react to reactogenic liposomes (with 1 exception out of >100 pigs over 8 years of experimentation using different pig strains and batches in the USA and Hungary), an average of only 5–7% of humans develop significant HSRs to reactogenic liposomal drugs. Thus, pigs present a good model of only those (5–7%) humans who do react to Doxil.

As for liposome reactions in rats, a series by Rabinovici et al. evaluated the hemodynamic, biochemical, and hematological responses of Sprague Dawley rats to DSPC/DMPG/Chol/α-tocopherol (47:5:47:1 mole ratio) large unilamellar liposome (LUV)-encapsulated hemoglobin (LEH) and corresponding LUV. The authors “top-loaded” the animals with 0.7–1.7 g/kg LEH phospholipid (5.2 ml/kg from a 40–100 mM phospholipid LEH stock to ~300 g rats), i.e., 4000 to 35,000-fold larger doses than the ED₅₀ of similar MLV in pigs, yet this huge dose caused “only” transient tachycardia, hypertension, thrombocytopenia, leukocytosis and hemoconcentration, as well as elevation of plasma TXB₂. In further studies using similar “top-load” model, we injected rats with DMPC/DMPG/Chol (50:5:45 mole ratio) MLV and LEH at 400 mg/kg phospholipid, which too was well tolerated despite massive C consumption and elevation of blood TXB₂ (Fig. 2; Szebeni et al., 1994). Likewise, Doxil boluses containing up to ~100 mg phospholipid/kg did not cause noticeable hemodynamic changes in Sprague Dawley rats (unpublished observations), which is consistent with the low sensitivity of this species to liposome-induced acute reactions.

As for the sensitivity of dogs to i.v. MLV or Doxil, preliminary, unpublished experiments suggest that this species is equally sensitive as pigs and reactive man. Importantly, unlike pigs and rats, dogs also react to micellar solvent systems, such as Cremophor EL (CrEL) and polysorbate 80 (tween-80; Lorenz et al., 1977; Poirier et al., 2004), thereby enabling the study of HSRs to the anticancer drugs Paclitaxel (Taxol) and docetaxel.
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(Taxotere) as well as to the hypnotic/anesthetic agents, Althesin, propandid and Stesolid (Szebeni et al., 1998, 2001). Fig. 3 shows the time course of two typical symptoms of (0.05 ml/kg) CrEL-induced HSR in dogs: systemic hypotension with paralleling leukopenia followed by leukocytosis. The most prominent blood cell change caused by Doxil in dogs is thrombocytopenia (unpublished observations).

**Figure 2.** Blood pressure changes with associated C activation and thromboxane B2 elevation in rats following injection of liposome-encapsulated hemoglobin (LEH). Modified from Szebeni et al. (1994). CH50, unit of C consumption; MBP, mean blood pressure.

**Figure 3.** Blood pressure and associated white blood cell changes (leukopenia followed by leukocytosis) in a dog administered 1 ml/kg Cremphor EL i.v. MAP, mean arterial pressure; WBC, white blood cells.
**Influence of Lipid Composition**

The cholesterol content of liposomes may be an important determinant of HSRs on the basis that the pulmonary hypertensive effect of MLV in pigs was found to be proportional with the amount of Chol in the vesicles in 20–71% range (Szebeni et al., 2000), and in rats, MLV containing 71% Chol were significantly more reactogenic compared to liposomes with 45% Chol (Baranyi et al., 2003). In the latter study, “only” 6 mg phospholipid/kg “high-cholesterol” MLV caused massive, tachyphylactic hypotension (Fig. 4) with decreased respiration rate (Fig. 5), a CARPA symptoms that had not been previously demonstrated in animals. Likewise, for the first time, the above study presented evidence of shock-lung and myocardial damage following bolus injection of “high-cholesterol” MLV, the most potent C activator liposome preparation in our hands to date (Baranyi et al., 2003).

**Conclusion**

Complement-mediated HSRs represent a poorly understood safety problem with state-of-art “nano-medicines”, including a variety of liposomal and micellar drugs. However, none of the existing in vivo immunotoxicology models are applicable to measure and predict these reactions (Bala et al., 2005; Nierkens and Pieters, 2005; Uetrecht, 2006). The animal models reviewed here, particularly the porcine “liposome-induced cardiopulmonary distress model” closely mimics the sensitivity and symptoms of human liposome reactions, thus it provides a useful preclinical model for safety screening and mechanistic studies in this context.

**Figure 4.** Blood pressure changes in a rat treated with high-cholesterol MLV (MLV containing 71% Chol). The arrows indicate repetitive administration of 6 mg/kg MLV boluses. The curve demonstrates the massive hypotensive effect of these liposomes and the gradual decrease of response, attesting to tachyphylaxis (i.e., response fatigue). Modified from Baranyi et al. (2003). MBP, mean blood pressure.
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area. CARPA can also be reproduced and quantitatively studied in dogs, while the high tolerance of rats to particulate lipids lessen their value as a model system.

Abbreviations

C complement
CAS cardiac abnormality score
Chol cholesterol
CO cardiac output
DMPC Dimyristoyl phosphatidylcholine
DMPG dimyristoyl phosphatidylglycerol
ECG electrocardiography
HSRs hypersensitivity reactions
LUV large unilamellar vesicles
MAP mean arterial pressure
MBP mean blood pressure
MLV multilamellar vesicles
PAP pulmonary arterial pressure
SAP systemic arterial pressure
TXB2 thromboxane B2

Figure 5. Respiration of a rat during high-cholesterol MLV-induced HSR, illustrating liposome-induced decrease of respiration rate, a model of HSR-related apnea. Modified from Baranyi et al., (2003).

References


