

Complement Activation Related Pseudoallergy and Its Animal Models

PhD thesis

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Abbreviations

Ab - antibody

ADCC – antibody-dependent cell-mediated cytotoxicity

Ag - antigen

C - complement

CARPA – complement activation related pseudo-anaphylaxis

CH – complement hemolysis

CO – cardiac output

CR – complement receptor

CrEL – chromophor-EL

CVP – central venous pressure

CVR – coronary vascular resistance

DAF – decay accelerating factor

DLS – dynamic light scattering

ELISA – enzyme linked immunosorbent assay

ETCO₂ – end-tidal carbon dioxide

FDA – United States Food and Drug Administration

GBM – glomerular basal membrane

HR – heart rate

IgE – immune globulin E

IgG – immune globulin G

IgM – immune globulin M

i.v. – Intravenous

LPS – Lipopolysaccharide

LVEDP – left ventricular end diastolic pressure

M – mast cells

MAC – membrane attack complex

MASP – mannose-binding lectin associated serine protease

MBL – mannose-binding lectin

MLV – multilamellar vesicle

NHS – normal human serum

NS – normal saline

PAP – pulmonary arterial pressure

PEG – polyethylene glycol

PEI – polyethylene imine

PIM – pulmonary intravascular macrophage

PL – phospholipid

PMN cells – polymorphonuclear cells

PVR – pulmonary vascular resistance

RBCs – red blood cells

SAP – systemic arterial pressure

SRBC – sheep red blood cells

SVR – systemic vascular resistance

TCC – terminal complement complex

Tc cells – cytotoxic T cells

Th1 cells – T helper 1 cells

TXA₂ – thromboxane A₂

USP – United States Pharmacopeia

WBCs – white blood cells

Introduction

A large variety of chemical substances as well as medicinal products have been suspected or proven to provoke adverse immunological reactions. One of the most common side effects of drugs is hypersensitivity reaction that often prevents or limits their use. These reactions also have been a major cause of drug withdrawal from the market in the past few decades. Their prevention and treatment is of utmost importance.

Hypersensitivity, anaphylaxis, pseudo-anaphylaxis

Hypersensitivity means adverse reactions of the normal immune system. These range from a mild localized rash to detrimental effects on vital systems. The classification by Coombs and Gell distinguishes 4 types^{1,2} (Table 1):

Type 1 – Allergy (immediate), mediated by IgE (and IgG4)

Type 2 – Cytotoxic, antibody-dependent, mediated by IgM or IgG (complement)

Type 3 – Immune complex disease, mediated by IgG

Type 4 – Delayed type hypersensitivity, T-cell-mediated immune memory response, antibody-independent.

+1 – Sometimes used as a distinction from type 2. Instead of binding to cell surface components, the antibodies recognize cell surface receptors. Often these reactions are classified as type 2, or a subcategory of type 2.

Table 1. Characteristics of the four classical types of hypersensitivity reactions.

| | Type I | Type II | Type III | Type IV |
|-------------------|--|--|--|--|
| Synonym | IgE mediated hypersensitivity, Anaphylaxis | IgG mediated hypersensitivity, Cytotoxic | Immune complex mediated hypersensitivity | Cell mediated hypersensitivity |
| Mechanism | Ag induces crosslinking of IgE bound to mast cells and basophiles with release of vasoactive mediators | Ab directed against cell surface antigens mediates cell destruction via complement activation or ADCC | Ag-Ab complexes deposited in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils | Sensitized Th1 cells release cytokines that activate macrophages or Tc cells which mediate cellular damage |
| Clinical examples | Typical manifestations include systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, food allergies, and eczema | Typical manifestations include blood transfusion reactions, erythroblastosisfetalis, and autoimmune hemolytic anemia | Typical manifestation include localized Arthus reaction such as serum sickness, necrotizing vasculitis, glomerulonephritis, rheumatoid arthritis, and systemic lupus erythematosus | Typical manifestations include contact dermatitis, tubercular lesions and graft rejection |
| Time elapsed | Seconds to minutes | Hours to a day | Hours to days | 2-3 days |
| Cell pathology | Accumulation of neutrophils, eosinophils. Smooth muscle contraction | Phagocytosis, lysis of target (RBCs, platelets, WBCs, GBM) | Accumulation of neutrophils, macrophages. Release of lytic lysosomal enzymes | Lymphocytes, and macrophages; granulomas |

Anaphylaxis is a severe, potentially life-threatening hypersensitivity reaction to an allergen. The first documented individual who developed an anaphylactic reaction was Pharaoh Menes who died from a wasp sting in 2640 BC³. Many years later, in 1902, Portier and Richet used the term anaphylaxis to describe acute reactions developing in dogs after repeated injections of the sea anemone toxin⁴.

Upon first exposure to a substance the immune system becomes sensitized, causing an allergic reaction to occur on repeated exposure. During this quick reaction histamine and other mediators are released, symptoms develop rapidly, within seconds or minutes. These include abdominal pain and cramping, abnormal breathing, wheezing, anxiety, confusion, cough, diarrhea, difficulty swallowing, fainting, light-headedness, dizziness, hives, itchiness, nasal congestion, nausea, vomiting, palpitations, skin redness, rash, mottling, slurred speech. Further clinical signs are arrhythmia, pulmonary edema, hypotension, and angioedema. Anaphylaxis is an emergency condition and requires immediate treatment, often tracheostomy, endotracheal intubation, epinephrine, antihistamines and corticosteroids. In the most severe cases airway occlusion, respiratory and/or cardiac arrest and shock can develop. Common causes include drug allergies, food allergies, insect bites or stings.

Pseudo-anaphylaxis has very similar clinical symptoms as anaphylaxis, without detectable immunological sensitization (antibodies or sensitized cells). It is also called pseudo-allergic or anaphylactoid reactions. “Non-immune anaphylaxis” is the term currently used by the World Allergy Organization for classification, although this nomenclature is somewhat misleading, as for example the complement system, which is often involved in these reactions, is part of the immune system.

The mechanisms of these reactions are not well understood and include direct liberation of vasoactive mediators (e.g. histamine), general mast cell or basophil activation with release of other mediators, activation of the complement or other plasma protein systems (coagulation, kinin-kallikrein) as well as neuro-psychogenic reflex mechanisms.⁵

Classical hypersensitivity requires a pre-sensitized state of the immune system. However, in some cases adverse immune reaction can occur on the first exposure to the

drug.⁶ Unlike IgE-mediated allergy, these reactions arise without prior sensitization and symptoms often lessen or disappear on later treatments (Table 2).

Possible mechanisms of pseudo-allergic reactions:

- Direct release of mediators (e.g. histamine)
- Direct activation of complement system
- Activation of the coagulation system
- Interaction with kinin-kallikrein system
- Shift in eicosanoid metabolism toward leukotriene formation
- Platelet activation
- Psycho-neurogenic reactions

Table 2. Differences between classical anaphylaxis and pseudo-anaphylactic reactions

| | Anaphylactic reactions | Pseudo-anaphylactic reactions |
|---|------------------------|-------------------------------|
| Is sensitization required? | Yes | No |
| Can reaction occur on first exposure? | No | Yes |
| Is reaction predicted by allergy skin test? | Yes | No |

Complement system

The complement system is part of the humoral arm of the innate, nonspecific immune system (Figure 1). It consists of about 30-35 proteins, some bound to cell membranes and others found in the blood plasma, most of which are synthesized by the liver. A number of these proteins circulate as inactive precursors and form a cascade that can be activated typically by antibody bound to an antigen.

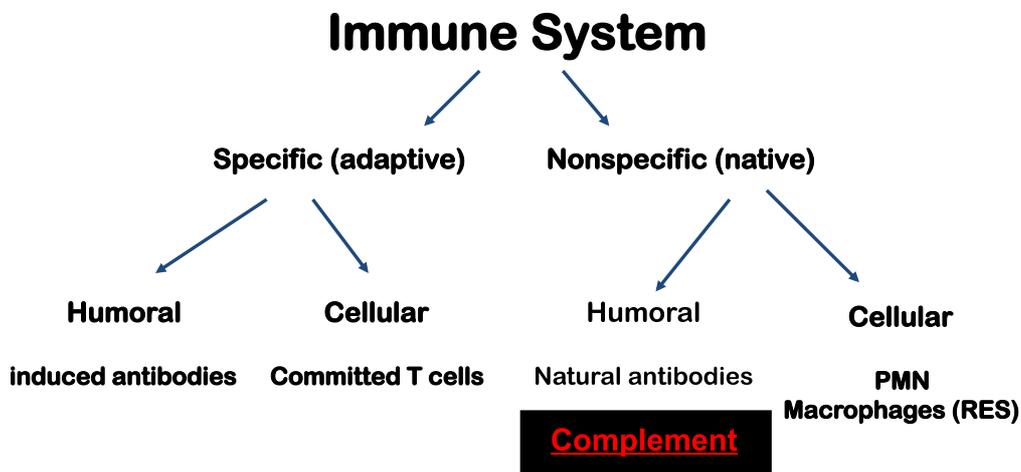


Figure 1. Components of the immune system

The purpose of the complement system is to assist, or “complement” the action of antibodies in defense against bacteria and rid the body of antibody-coated antigens (antigen-antibody complexes). When the complement cascade is triggered, activation of one of the three pathways leads to the formation of the terminal complement complex (TCC), along with the generation of anaphylatoxins (C5a and C3a) and the release of vasoactive mediators.

The terminal complement complex – also known as membrane attack complex (MAC) – has the conformation of a cylinder that is inserted into the cell wall/membrane of the bacteria causing the bacterial cells to swell, burst, and die. Other elements of the

complement system act as opsonins or chemo-attractants, making the recognition and clearance of the pathogens by other components of the immune system more efficient.

The three pathways that activate the complement system are the classical complement pathway, the alternative complement pathway and the mannose-binding lectin pathway (Figure 2). They function as an enzymatic cascade: inactive proteases are cleaved to form activated proteins with the capacity to cleave downstream proteins.⁷

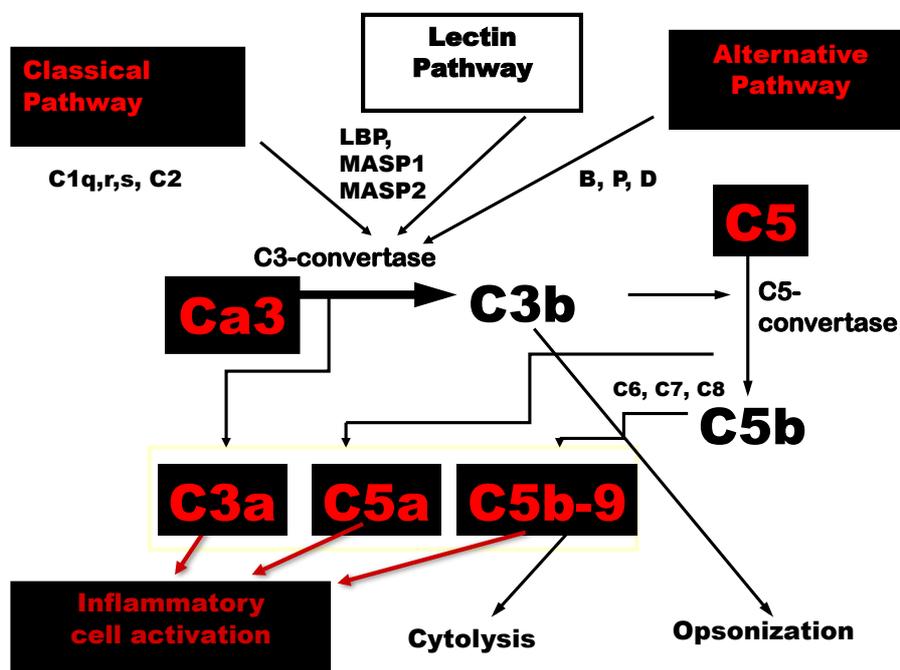


Figure 2. The most important complement factors and the three complement activation pathways

Activation of the classical pathway is initiated by specific binding of the component C1q to the Fc region of the antibodies that are present in immune complexes or on the surface of pathogens. C1q cannot be directly activated by free antibodies, only by antibodies bound to various sites, such as on the surface of pathogens. IgM, then IgG1 and IgG3 are the most effective antibodies at activating the complement. C1q is activated after binding to the Fc region of antibodies and in turn activates the normally inactive serine proteases C1r and C1s. C1s interacts with C4 to form C4a and C4b, then with C2 which is cleaved into C2a and C2b. C2b and C4b form the complex C4bC2b, which is the classical C3 convertase.

The extent of complement activation by the classical pathway is under control of the C1 inhibitor, which dissociates C1r and C1s from C1q, and inactivates the spontaneous low activation of C1q. Other regulatory proteins include the C4-binding protein that controls activation of the classical C3 convertase.

The alternative pathway is initiated by the spontaneous cleavage of C3 - the most abundant complement protein in the plasma - to C3(H₂O) which binds to factor B. Factor B is then cleaved to Bb by factor D. C3(H₂O)Bb is a soluble convertase which cleaves C3 into C3a and C3b. A fraction of formed C3b binds to factor B, which is then cleaved to Ba and Bb by factor D. C3bBb is the alternate C3 convertase.

Regulatory proteins of the alternative pathway are found either in the plasma or on cell membranes. Complement receptor 1 (CR1) and the decay-accelerating factor (DAF or CD55) compete with factor B to prevent its binding to C3b. Factor I together with CR1 and the membrane co-factor of proteolysis (MCP or CD46) cleave C3b to the inactive protein iC3b. Finally, factor H binds to C3b and prevents the formation of C3bBb by competing with Bb.

Activation of the third, mannose-binding lectin pathway is initiated by a protein very similar to C1q, the mannose-binding protein, which binds specifically to mannose and other sugars on the surface of pathogens. In turn, the bound protein forms a complex with two proteases very similar to C1r and C1s, MASP1 and MASP2, which are activated to cleave C4 and C2 with the resulting formation of the C4bC2b convertase.

One result of complement activation is opsonization. C3b is covalently bound to the surface of pathogens, and is recognized by phagocytes via CR1. A large amount of C3b can be deposited on the surface of pathogens, facilitating uptake and elimination.

Another result is the generation of anaphylatoxins, i.e. C3a, C4a and C5a. These activate mast cells and basophils causing the release of inflammatory mediators, such as histamine, thromboxane, tryptase, etc.

Another major consequence is the formation of the membrane attack complex (MAC). The binding of C3b to C4bC2b or Bb produces the C5 convertase, which cleaves C5 to C5a and C5b. C5b initiates the assembly of the MAC and results in the direct lysis of the pathogen cells.

Activation of the complement system also enhances the removal of circulating immune complexes that cannot be engulfed by phagocytes. The number of IgG molecules in immune complexes is sufficient to bind and activate C1q, then C4 and C3. Immune complexes tagged with C4b and C3b are bound to CR1 on the surface of erythrocytes so that macrophages in the spleen and the liver can degrade immune complexes.

CARPA

Szebeni et al. coined the term complement activation related pseudo-anaphylaxis for this new class of hypersensitivity reaction in 1999⁸, when they examined the role of complement in pseudo-allergic cardiopulmonary reactions to intravenously administered liposomes. By the time of those studies 4 liposomal drugs encapsulating doxorubicin, daunorubicin, and amphotericin B were already in clinical use in several countries, and many other liposomal preparations were in advanced stages of clinical trials.⁹ Adverse events reported during the use of these formulations draw attention because of their unusual characteristics. Some of these infusion reactions appeared on the first exposure to the drugs, immediately after the start of the infusion, and symptoms included dyspnea, tachycardia, chest pain, hypotension or hypertension, and back pain. The reactions were categorized as pseudo-allergy, to distinguish these hypersensitivity reactions from the classical IgE mediated allergy.¹⁰⁻¹⁵

The relatively high frequency of such reactions was also worrying. Among 705 patients treated with Doxil 6.8% exhibited symptoms of some degree of pseudoallergy.¹⁶ The underlying mechanism being unknown, it was impossible to specifically predict, prevent, or treat these reactions, some of which were severe, occasionally life threatening, and excluded these patients from further therapy with these drugs.

Based on the fact that certain liposomes can activate the complement systems¹⁷ and that complement activation can lead to similar cardiovascular and pulmonary symptoms as described above^{18,19}, the authors investigated the possible involvement of the complement system in these hypersensitivity reactions provoked by liposomal drug delivery systems.

Szebeni et al. proved that the complement system has a causal role in the cardiopulmonary distress exhibited in this porcine model of pseudo-allergy and a hypothetical reaction sequence (Figure 3) was described explaining hemodynamic changes following intravenous injection of liposome boluses in the pigs.

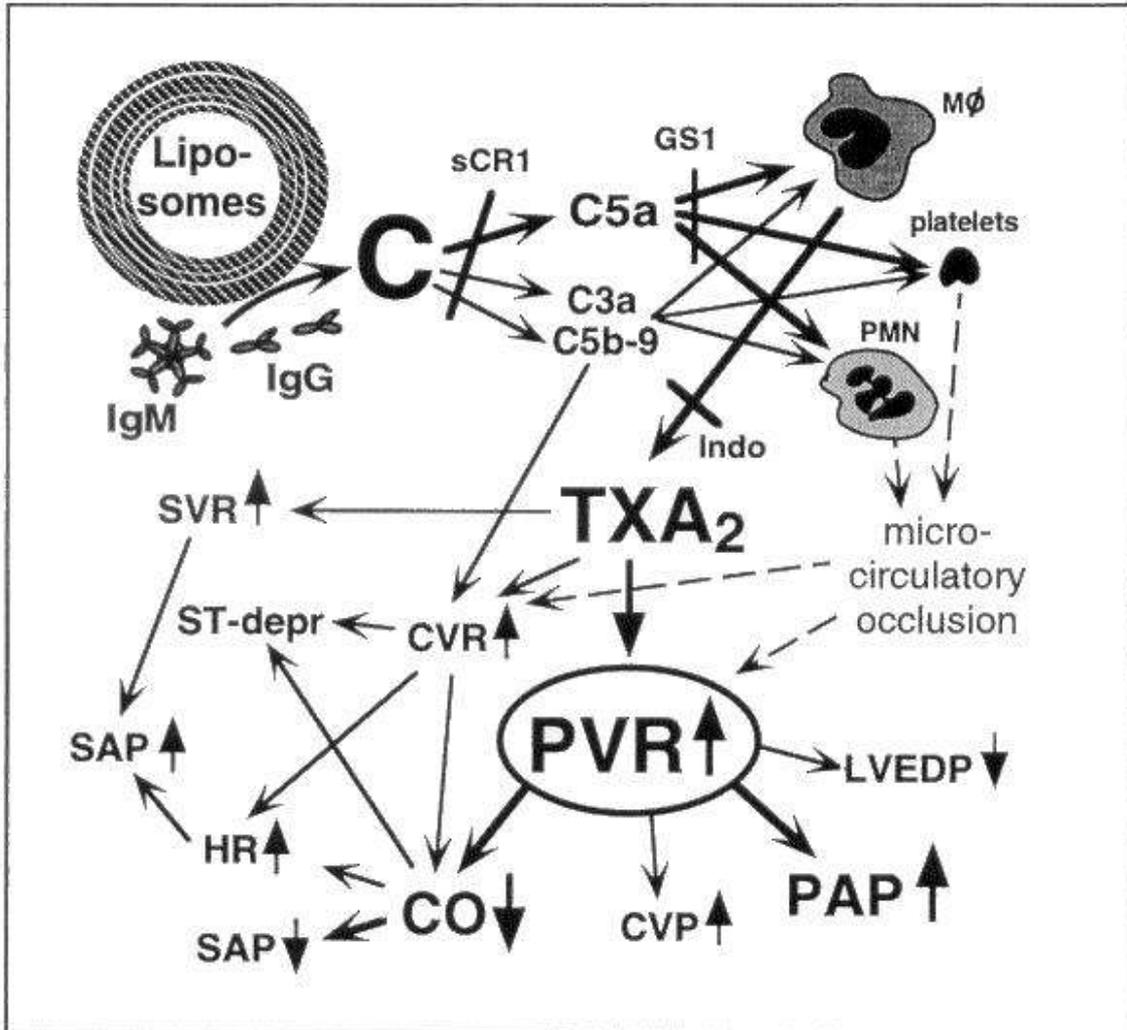


Figure 3. Liposomes recognized by IgM and IgG activate the complement system (C) and anaphylatoxins are formed (C5a and C3a), along with the assembly of the membrane attack complex (C5b-9). Anaphylatoxins activate mast cells (M), platelets and polymorphonuclear cells (PMN). As a result thromboxane (TXA₂) is released from mast cells, and the platelets and PMN form occlusions in the microcirculation of the lung. The coronary vascular resistance (CVR) and the pulmonary vascular resistance (PVR) and pulmonary arterial pressure (PAP) increases, causing ischemia and acute pulmonary hypertension. The central venous pressure (CVP) increases, the left ventricular end diastolic pressure (LVEDP) drops. These all lead to compromised cardiac output (CO) with diminished systemic circulation and systemic arterial pressure (SAP). In some cases the consequential compensatory mechanisms, i.e. increase in heart rate (HR) and in systemic vascular resistance (SVR) may result in elevated SAP.

Drugs activating the complement system

In the past decade several drugs and chemicals were shown to have a potential to trigger complement activation related pseudo-anaphylaxis (CARPA). These include but are not limited to particulate radio contrast media²⁰, drug delivery systems, carbon nanotubes²¹, liposomes²² and micellar solvents²³, such as Cremophor EL (CrEL) in Taxol²⁴. The monitoring of CARPA became an important aspect in the development of these pharmaceuticals. Underlying the importance of this new type of hypersensitivity, *in vitro* and *in vivo* testing of complement activation became a recommended toxicology test by the US Food and Drug Administration²⁵.

Nanomaterials are expected to revolutionize materials science, technology and a wide range of industries, including medicine. By controlling the structure of materials on a super-fine scale, nanotechnologies will improve functions and characteristics of materials as well as creation of new functions²⁶. By definition, a nanoparticle is a particle having one or more dimensions of the order of 100nm or less. Some representatives of this class are liposomes and polymers. There is an extensive amount of evidence attesting that the infusion of nanoparticulate systems, including regulatory-approved stealth nanomedicines, in some individuals is associated with cutaneous, respiratory and circulatory disturbances.^{27,28}

The harmful effects of nanoparticles arise from the combination of various factors, two of which are particularly important: (i) the high surface area, and (ii) the intrinsic toxicity of the surface²⁹. In contrast with conventional particles of larger mean diameter, nanoparticles under 100 nm can potentially be more toxic to the lung (portal of entry), can redistribute from their site of deposition, may escape from the normal phagocytic defenses and can modify the structure of proteins. Therefore, nanoparticles can activate inflammatory and immunological responses and may affect the normal tissue function.

Recently, the critical parameters determining the toxicity of nanoparticles have been proposed³⁰:

- Particle size, size distribution, shape, surface area, redox potential and properties, purity, identity of contaminants, catalytic activity and generation of reactive oxygen species;
- Interaction with biologically critical macromolecules such as DNA, membranes and cytoskeleton elements;
- Potential for unintended carriage of toxic molecules (toxic chemicals that may be present in the environment and are loaded along with therapeutic drugs on the surface of the nanoparticles);
- Nanoparticle escape from the normal phagocytic defences and redistribution from site of deposition (translocation);
- Agglomeration state (pro-agglomeration factors, size, structure and toxic effects of nanoparticle agglomerates before and after biomodification);
- Chemical composition (surface charge, shape, area, reactivity and solubility).

Liposomes

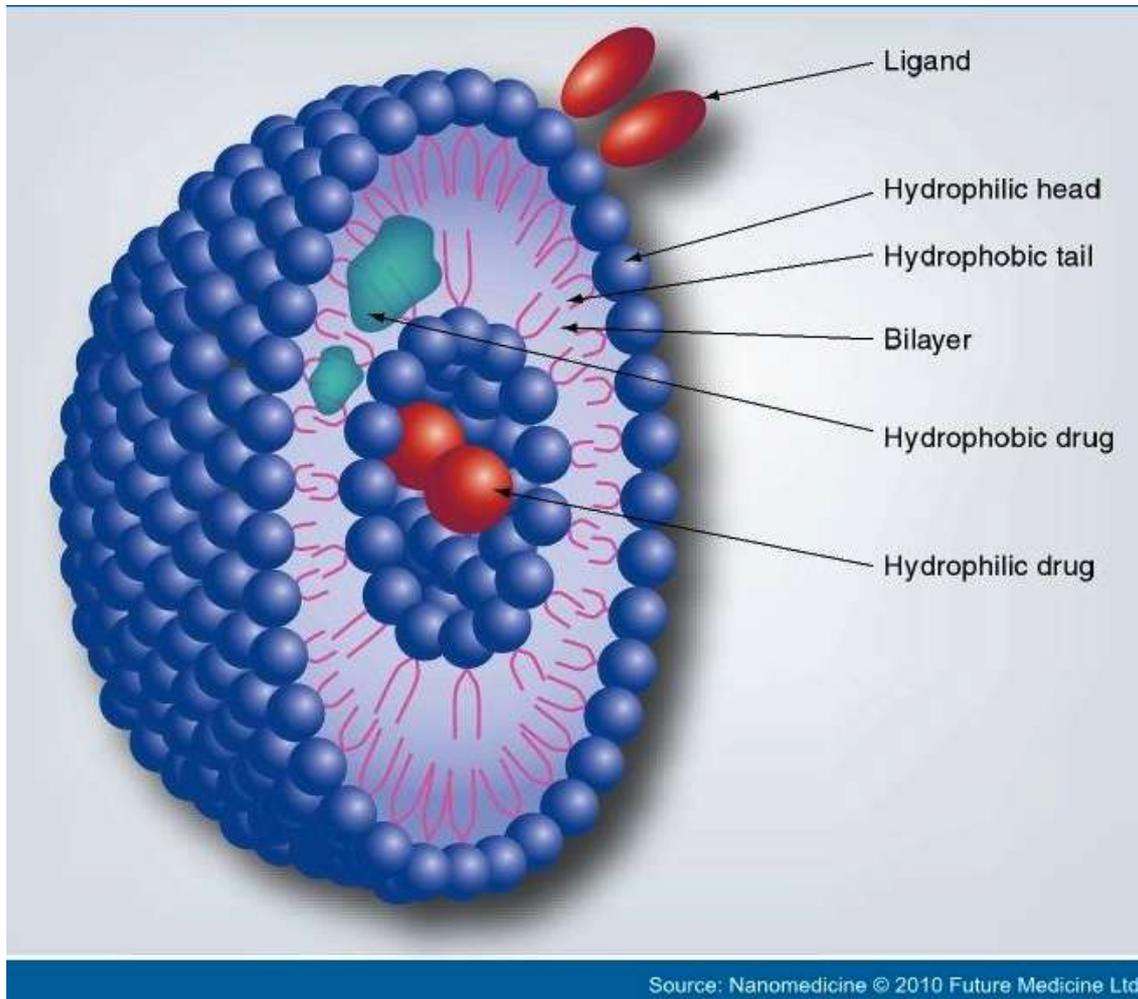


Figure 4. Cross section of a liposome

Since their discovery in the 1960s liposomes were used as a model to study biological membranes and as drug delivery systems for both hydrophilic and maybe even more importantly, for hydrophobic molecules. They are vesicles made of phospholipid bilayers, and can contain small amounts of other molecules, e.g. cholesterol (Figure 4). The three types of liposomes are small unilamellar liposomes and large unilamellar liposomes consisting of one bilayer, while multilamellar vesicles are formed by multiple bilayers of phospholipid molecules. Their clinical utility comes from the amphiphilic characteristic of phospholipids. Because of this, liposomes can carry both hydrophilic and hydrophobic molecules, and can even be targeted by surface functionalization, making them an ideal candidate for use as drug delivery systems.

However, shortly after their discovery it became apparent, that their similarity to biological membranes, a characteristic that provides opportunities to use them as a model system, can as well lead to the activation of the immune system.³¹ On one hand, this potential can be harvested with the use of liposomes as immune adjuvants.³² On the other, unintended hypersensitivity reactions can be detrimental for their use as drug delivery systems.²²

Polymers

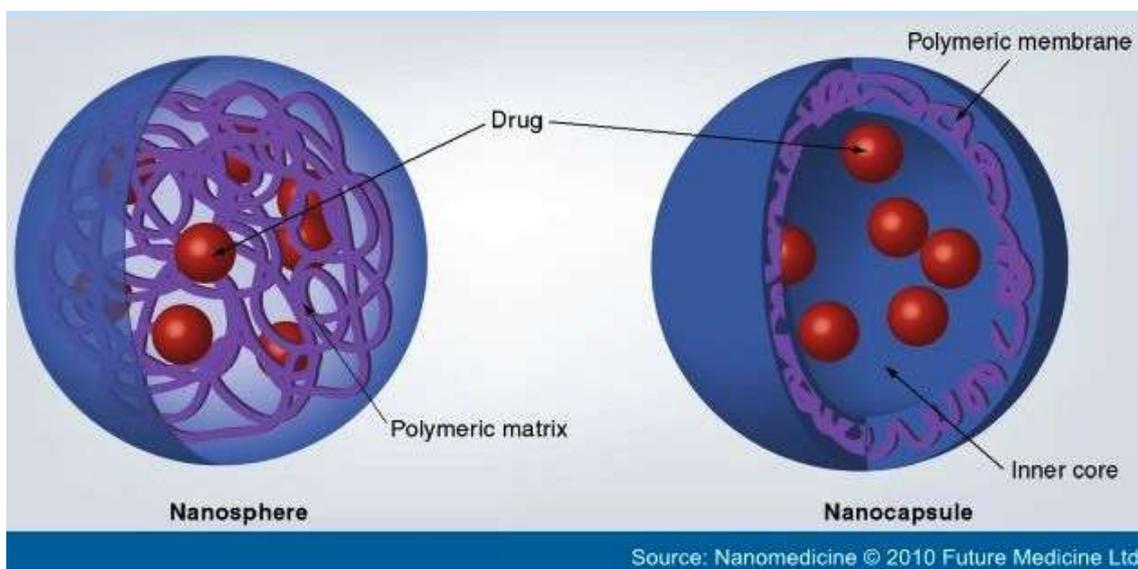


Figure 5. Polymeric nanoparticles

'Polymer therapeutics' (Figure 5) is a collective term used to describe polymeric drugs, polymer–drug conjugates, polymer–protein conjugates, polymeric micelles to which a drug is covalently bound, and multicomponent polyplexes that are being developed as non-viral vectors.³³ The idea of pairing up a bioactive agent, the drug proper with a carrier and transport vehicle dates back to the 1970s. Utilization of the nearly infinite compositional versatility of synthetic macromolecules and the potentials of synthetic chemistry allows tailoring of these drug delivery systems based on need.

Polymeric drug delivery systems may feature (i) subunits facilitating cell entry;

(ii) other subunits equipped with intra- or extrachain water-solubilizing groups; (iii) still other units acting as a homing device capable of directing the polymer-drug conjugate to the target tissue; and (iv), most importantly, units equipped with functional groups suitable for the critical conjugation step involving bioreversible drug binding to the polymer.³⁴

In vitro testing of the complement system

A detailed understanding of the interaction between nanomaterials/nanoparticles and the complement system is essential, as it could lead to innovation in the design and engineering of safer nanomedicines as well as development of safe clinical practices for their application.

Indications for the assays of complement system:

- Clarification of complement defects
- Detection of activation of the system
- Verification of adequate regulation

In the context of nanomaterial and nanomedicines safety, the most important is the monitoring of complement activation. There is a wide range of tests currently used for biocompatibility testing.

In the static *in vitro* model test materials are incubated with serum (e.g. NHS = normal human serum, defined and complement-tested standard serum) under certain circumstances (e.g. 37 °C water bath) for a defined period of time (e.g. 1 hour). The reaction is stopped and complement activation products (C3a, C5a, C4d, Bb, SC5b-9) are measured (e.g. with ELISA). These are highly sensitive assays and identification of various activation products helps differentiation between the activation pathways involved. C4d and Bb measure activation via the classical and the alternative pathway, respectively, and SC5b-9 is a factor representing the common terminal part of all the activation chains.

A similar test can be carried out with whole blood. The presence of blood cells provides an environment that models the conditions found in the human circulation closer than serum or plasma tests. However, exceptional care should be exercised when using anticoagulants, as these can have significant effect on the complement cascade. EDTA and citrate may inhibit complement activation; heparin has inhibitory or

stimulating effect depending on its concentration. Lepirudin (hirudin) is the only anticoagulant that has no known impact on the complement activation.³⁵

These kits may also be used for *ex vivo* detection of complement factors and cleavage products in samples taken from individuals exposed to the drugs or test substances prior to collection.

Conversely, analysis of animal serum and plasma samples is more challenging. There are only a very limited number of assays available for the detection of animal complement factors and activation products. Rat and mouse C5a ELISA kits are commercially available, and methods for the quantification of porcine C5a have also been described in the literature³⁶. However, the use of C5a as a marker for *in vivo* C activation is problematic because of the rapid clearance of C5a from blood by C5aR-carrying cells (WBC, platelets, macrophages, etc.), while specific ELISAs for the measurement of stable byproducts are not available.

The classical C hemolytic (CH50) assay fills this gap as it measures total hemolytic activity in a species-independent manner. The traditional CH50 is a measure of total complement activation through the ability of the complement system to lyse sensitized sheep red blood cells. The CH50 reflects the ability of the complement in the sample to activate. However, this is only an indirect measure of activation. When testing serum samples drawn from subjects, the CH50 measures the remaining complement activation capability of the sample, giving indirect information about the consumption of the complement factors in the subject. Hence the more activation in the assay, the less of the complement factors was consumed prior to the collection of the sample.

With the use of specific complement depleted sera, the test can be made more specific, focusing on a single protein. These assays are called CxH50, detecting the functional activity of a specific complement component in the sample, for example C3H50 using C3 depleted serum for the detection of C3 function in the sample.

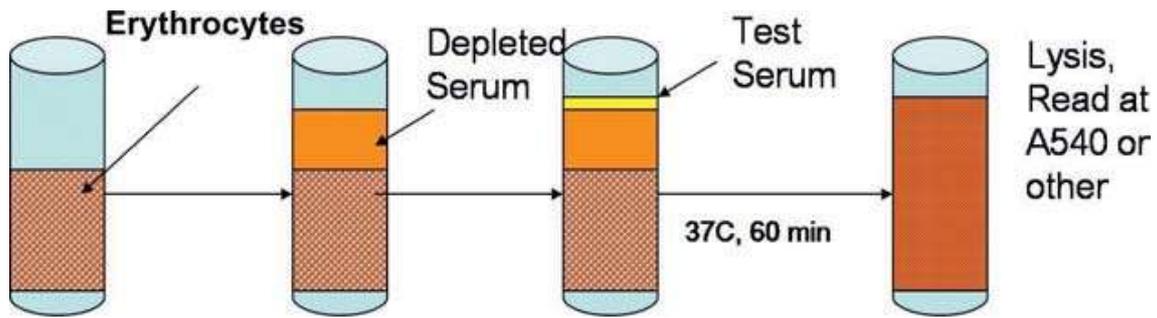


Figure 6. Principle of C_xH_{50} test

The CH_{50} is a screening assay for the activation of the classical complement pathway and it is sensitive to the reduction, absence and/or inactivity of any component of the pathway. The CH_{50} tests the functional capability of serum complement components of the classical pathway to lyse sheep red blood cells (SRBC) pre-coated with rabbit anti-sheep red blood cell antibody (hemolysin). When antibody-coated SRBC are incubated with test serum, the classical pathway of complement is activated and hemolysis results. If a complement component is absent, the CH_{50} level will be zero; if one or more components of the classical pathway are decreased, the CH_{50} will be decreased. A fixed volume of optimally sensitized SRBC is added to each serum dilution. After incubation, the mixture is centrifuged and the degree of hemolysis is quantified by measuring the absorbance of the hemoglobin released into the supernatant at 540nm. The amount of complement activity is determined by examining the capacity of various dilutions of test serum to lyse antibody coated SRBC.

The assay can be modified to measure the activity of a specific complement factor in the test serum. In the C_xH_{50} test the test serum is added to a serum depleted of the complement component of interest (C_x). (Figure 6)

In vivo models of CARPA

Animal models are the closest experimental alternatives to the human conditions. The interaction of the complex homeostatic systems is kept intact, allowing the analysis of physiological and pathophysiologic processes as they occur in a living organism. Animals, however, are not exactly like humans, and the selection of the appropriate model, and understanding of its limitations is of great importance. Care should be exercised with the interpretation of the results in order to correctly relate the findings to the human situation.

The advantage of rodents like mice and rats for complement research is the availability of biochemical assays for a wider range of complement factors and activation products. Also, genetically engineered animals, like knock out mice have potential utility for investigation of specific proteins, like complement factors and regulators.³⁷ However, phylogenetically these species are farther from humans. Additionally, due to their size, special equipment is required, and their low blood volume limits repeated blood sampling.

Non-human primates are the species developmentally closest to humans, however, their use raises some unique challenges due to legal limitations and safety, ethical, and financial considerations^{38,39}. In some cases their utility as research animals is also limited compared to other species.⁴⁰

Other larger animals used in research include cats, dogs and swine. Pigs are useful and relevant laboratory animals for several reasons. There is high sequence and chromosome structure homology between pigs and humans, indicating that the majority of the orthologous genes are conserved between the two species.⁴¹ Also, due to the size of the pig, instrumentation, monitoring, repeated blood sampling and pharmacokinetics closely resemble standard human treatment. Furthermore, the anatomy and physiology of pigs, that is particularly important for the study of the cardiovascular system and hemodynamics, is also very similar to humans.⁴² Considering these advantages and limitations, we decided to use the pig as a model for our in vivo studies.

Objectives

The endeavors to monitor and understand complement-activating properties of various drugs, implants, etc. may eventually provide laboratory tests for screening individuals and predicting risk of CARPA in patients who need advanced medical interventions utilizing nanomedicines, functionalized particulate systems and biomaterials. Furthermore, if effective preventive methods would be developed to avoid adverse events associated with immune-reactogenicity of certain drug delivery systems, even sensitive individuals might be able to benefit from these state of the art pharmacotherapies.

In our attempt to study complement activation by various nanoparticles and improve the safety and utility of these compounds, we had the following specific aims:

1. Establish an in vivo animal model for the testing of complement activation related pseudo-anaphylaxis by intravenously administered substances.
2. Identify the benefits of the in vivo model compared to in vitro complement testing.
3. Examination of the hemodynamic effects of serial intravenous injections of Doxil, a clinically used liposomal drug.
4. Based on the observations during in vivo testing of Doxil, develop a method for the prevention of complement activation related pseudo-anaphylaxis to the drug.
5. Evaluate the differences between in vitro and in vivo methods for the prediction of complement activation related pseudo-anaphylaxis provoked by polyethylene imine polymer nanoparticles.
6. Test utility of alternative animal species for studying complement activation by nanomedicines.

Methods

Preparation and characterization of test substances

Doxil

Commercial Doxil (Ben Venue Laboratories, Inc.) was obtained from the pharmacy of Semmelweis University. It contains doxorubicin HCl, 2 mg/ml, 4.22 mM; fully hydrogenated soy phosphatidylcholine (HSPC), 9.58 mg/ml; cholesterol (Chol), 3.19 mg/ml; N-carbamyl-poly(ethylene glycol methyl ether)-1,2-distearoyl-sn-glycerol-3-phospho-ethanol-amine triethyl ammonium salt with a polyethylene glycol (PEG) moiety of 2000 Da (2K-PEG-DSPE), 3.19 mg/ml; ammonium sulfate (≈ 0.2 mg/ml); histidine, 10 mM, pH 6.5, and sucrose, 10%. Total phospholipid content of Doxil is 12.8 mg/ml (13.3 mM).

Doxebo

Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), cholesterol (Chol), and egg yolk phosphatidylcholine (EPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama). Fully hydrogenated soy phosphatidylcholine (HSPC) and soy phosphatidylglycerol (HSPG) were from Lipoid Inc. (Ludwigshafen, Germany). All lipids had a purity of $\geq 97\%$. The negatively charged N-carbamyl-poly(ethylene glycol methyl ether)-1,2-distearoyl-sn-glycerol-3-phospho-ethanolamine triethyl ammonium salt (PEG-DSPE), having PEG moieties of 350 Da, 2 kDa, and 12 kDa (0.35K-PEG-DSPE; 2K-PEG-DSPE; 12K-PEG-DSPE, respectively), and the uncharged 3-methoxy polyethylene glycol-oxycarbonyl 3-amino-1,2-propanediol distearoyl ester having a PEG moiety of 2 kDa (2K-PEG-DS), were from Alza Corp. (Mountain View, California). The uncharged 3-methoxy-polyethelene glycol 1,2-distearoyl glycerol (2K-PEG-DSG) was from NOF Corp. (Tokyo, Japan).

The freeze-dried lipid components (originally dissolved in tertiary butanol) were hydrated in 10 ml sterile pyrogen-free normal saline (NS) by vortexing for 2-3 minutes at 70°C to form multilamellar vesicles (MLVs). The MLVs were downsized through 0.4- and 0.1- μ m 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 0.5 M NaCl/5 mM histidine buffer (pH 6.5). Micelles were prepared by extensive vortex mixing of 2K-PEG-DS-PE or 2K-PEG-DS in saline at 2 mg/ml, followed by filtration through 0.22- μ m filters.

The phospholipid concentration of preparations was determined using a modification of Bartlett's procedure.⁴³ The procedure is based on determination of the level of phosphorus, the common denominator for all phospholipids. To be specific for phospholipids it is necessary to extract the phospholipids from all other compounds that contain inorganic or organic phosphorus. The majority of phospholipid classes used for the preparation of liposomes (with the exception of cardiolipin, which contains 2) contain exactly 1 mole of phosphorus per mole of phospholipid; therefore, the phospholipid concentration can be derived directly from a quantification of the lipid phosphorus content of the sample. Standards/samples in aqueous phase were adjusted to the final sample volume with highly pure H₂O based on the sensitivity range selected (Table 3).

Table 3. Total phospholipid determination at three sensitivity ranges

| Addition | Sensitivity range (nmol) | | |
|---------------------------------|--------------------------|------------|------------|
| | 20-500 | 8-150 | 4-70 |
| HClO ₄ ^a | 1.0 ml | 0.4 ml | 0.2 ml |
| H ₂ O | 3.3 ml | 1.2 ml | 0.6 ml |
| Ammonium molybdate ^b | 0.6 ml | 0.2 ml | 0.1 ml |
| Reducer ^c | 150 μ l | 50 μ l | 30 μ l |
| Final volume | 4.75 ml | 1.73 ml | 0.87 ml |

^a Reagent 1

^b Reagent 2

^c Reagent 3

A few acid-washed silicon carbide boiling stones (Thomas Scientific, Swedesboro, NJ) were added to each sample to ensure safe boiling and to prevent loss. HClO_4 (reagent 1) was added according to the selected sensitivity range. Samples were heated to the boiling point (180–200°C) for 30 min, and then cooled to room temperature. Water, then reagent 2, and then reagent 3 were added to fit the desired sensitivity range (Table 3) and mixed well after each addition. Immediate mixing is important to obtain low and reproducible blanks because ammonium molybdate may contain a small amount of phosphorus. The test tubes were heated at 100°C for 7 min, and cooled to room temperature. The samples were read in a spectrophotometer at 830 nm for high sensitivity or at 660 nm for low sensitivity, against the reagent blank. Each determination was performed in triplicate and included at least a partial calibration curve and reagent blanks.

Particle size distribution was determined by dynamic light scattering (DLS), using an ALV-NIBS/HPPS High Performance Particle Sizer with ALV-5000/ EPP multiple digital correlator (ALV-Laser Vertriebsgesellschaft GmbH, Langen, Germany). Liposome surface potential was determined by measuring 4-heptadecyl-7-hydroxycoumarin ionization over a broad range of pH values as described earlier.⁴⁴ Table 4 shows the essential characteristics of all preparations used. (Table 4)

Table 4. Physicochemical characteristics of preparations

*Mean size was determined by DLS in 5% (w/v) dextrose, triplicate measurements with $\text{SD} < 10\%$. ND, not done.

| Name | Lipid mole ratio | Mean size* (nm) | Surface potential (mV) | Zeta potential (mV) |
|--------|------------------|-----------------|------------------------|---------------------|
| Doxil | 57:38:5 | 108 | ND* | -13.3 |
| Doxebo | 57:38:5 | 124 | -52 | -10.1 |

In the results section, injected or infused doses of Doxil and Doxebo are given as mg phospholipid content per kg bodyweight. The doxorubicin : phospholipid w/w ratio for Doxil is 1 : 6.385.

Determination of bacterial endotoxin (LPS) in liposome dispersions

The LPS content of liposomes prepared for this study was determined by a Limulus amoebocyte lysate assay (PYROGENT Plus, Cat. No. N294-06, Cambrex Bio Science Walkersville, Inc., Walkersville, Maryland), after dissolving (96% ethanol) and separating (ultrafiltration using 20 kDa cutoff membrane) the lipids from LPS.⁴⁵ Acceptance criterion as pyrogen-free was ≤ 0.5 endotoxin units (EU)/ml (0.01-0.25 ng LPS/ml).

Morphological analysis of liposomes by differential interference contrast (DIC) and cryotransmission electron microscopy (cryo-TEM)

A light microscope with DIC (Nomarski) optics was used to examine the presence of aggregates in various liposome preparations. A small drop of the liposome stock solutions was placed in a concave well of a glass slide and covered with a coverglass. The cryo-TEM analysis of liposomes was performed by methods described earlier.^{46,47}

Polymers

A collaborator in Germany prepared the polymers used in our studies. Branched poly(ethylene imine) with a molecular weight of 25 kDa (PEI 25 kDa, PolyminTM) and a molecular weight of 5 kDa (PEI 5 kDa) were gifts from BASF (Ludwigshafen, Germany). The block copolymers poly(ethylene glycol)-graft- poly(ethylene imine) PEI(25k)-PEG(20k)₁ and PEI(25k)-PEG(2k)₁₀ were synthesized as described earlier.⁴⁸ Polymer solutions were prepared in endotoxin-free water and plastic ware (Pyroquant Diagnostik GmbH, Mörfelden-Walldorf, Germany). Schematic structures of the tested polymers and block copolymers are shown in Figure 24A.

USP bacterial endotoxins test of the polymers

The Limulus Amoebocyte Lysate (LAL) test kit Pyrotell™, Control Standard Endotoxin (CSE) and USP Sterile Water for Injection or Irrigation (WFI) were a gift from PyroquantDiagnostik GmbH, Mörfelden-Walldorf, Germany. The assay was performed according to the manufacturer's protocol. Briefly, polymer solutions were diluted to 10 mg/ml with WFI of which 100 µl was mixed with 100 µl reconstituted Pyrotell in the designated glass test tubes. A positive control of 2λ Control Standard Endotoxin (CSE) and a negative control of WFI were treated like the polymer samples. The mixtures were incubated for 60 min at 37°C before the test tubes were flipped upside down and checked for gel retention at the bottom of the test tube.

Zymosan

Zymosan was from Sigma Chemical Co. (St. Louis, Missouri). A stock solution with a concentration of 10 mg/ml was freshly prepared before the experiments with normal saline.

In vivo tests of complement activation related hemodynamic reactions*Instrumentation*

Experiments using pigs and dogs were performed at the Semmelweis Medical University in Hungary and at the Uniformed Services University of the Health Sciences (USUHS) in the USA, and were approved by the local Animal Subject Review Committees and followed their guidelines, treating the animals humanely.

Swine (25-40 kg) and mongrel dogs (20-40 kg) of both sexes were purchased from approved local vendors. They were sedated with i.m. ketamine (500 mg) and intubation was carried out with a 6.5 Fr tracheal tube to maintain free airway, and to enable controlled ventilation if needed. The animals were anesthetized with 1-2%

isoflurane, or with i.v.xylazine/ketamine mixture and Nembutal (pentobarbital, 30 mg/kg for induction and 5-10 mg/kg/h for maintenance), via the ear vein. 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 (during pentobarbital anesthesia) was assisted by a Harvard ventilator (Harvard Apparatus, Cambridge, MA).

Surgery was performed to cannulate the right external jugular vein for drug injections and a Swan-Ganz catheter was floated through the right atrium and right ventricle to the pulmonary artery for pulmonary arterial pressure (PAP) and central venous pressure (CVP) measurement. 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, CVP), heart rate (HR) and ECG were continuously monitored throughout the experiments. Additionally, continual respiratory rate, end-tidal carbon dioxide (ETCO₂), and rectal temperature were monitored with an M1026A Gas Analyzer and Model 68 clinical monitor (Hewlett-Packard, Andover, MD) when available.

Test materials were injected through the Swan-Ganz catheter into the pulmonary circulation and flushed with 5 ml saline solution. Between injections of test and/or reference material, a resting period of at least 20 minutes was maintained.

SAP, PAP, CVP, standard ECG leads I, II, and III and – instrumentation permitting - ETCO₂ were recorded continuously with the SPEL Advanced Haemosys data acquisition system (Experimetria Ltd., Budapest, Hungary) or an ADInstrumentPowerLab Recorder and LabChart™ software (ADInstruments, Bella Vista, NSW, Australia) at a sampling rate of at least 100 Hz. Other details of surgery, instrumentation and hemodynamic analysis were described previously^{8,50,51}. (Figure 7)

CARPA Setup

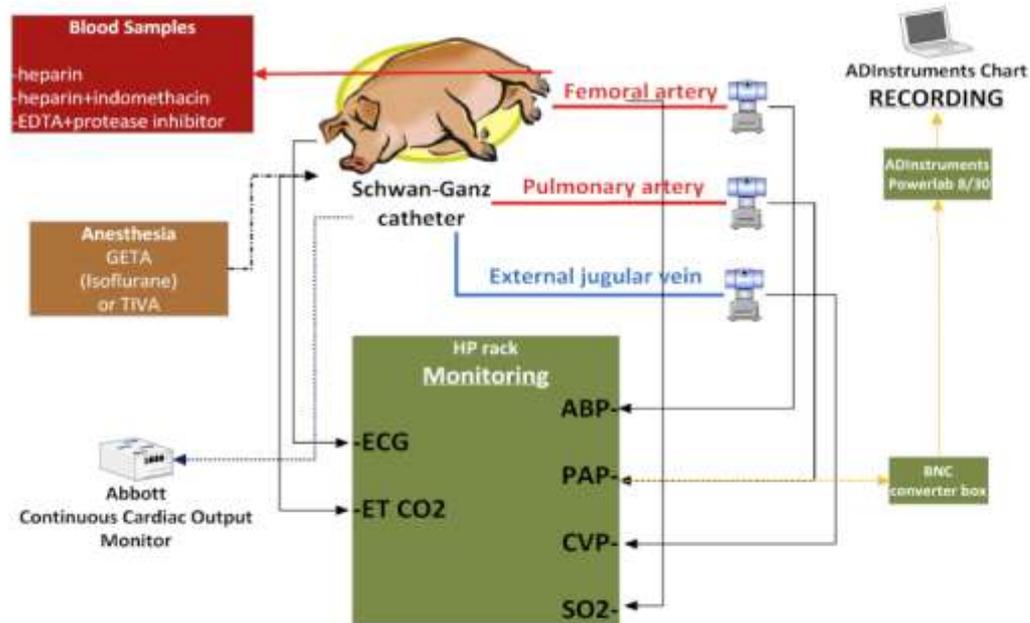


Figure 7. A schematic figure of the instrumentation setup, the monitored parameters, and the experimental endpoints in the swine model

Cardiopulmonary data analysis

The pulmonary and systemic blood pressure was registered continuously at 100-1000 Hz. We averaged the mean blood pressure every 30 seconds before and after the injections of the various substances and plotted against time. We also registered the maximal increase of mean pulmonary and systemic arterial pressure after each injection and compared the changes provoked by the various test substances. When comparing groups in the results section, the blood pressure values are expressed as medians.

Blood collection and analysis:

Blood samples of 4-6 ml were collected in tubes coated with EDTA (BD Vacutainer, BD Franklin Lakes, NJ USA). Samples were drawn at 0 (baseline) and at 3, 6, 9 and 12 minutes after each injection, leaving at least 20 minutes rest periods between

instrumentation and baseline sampling, and between injections.

Statistical analysis

The significance of PAP and SAP changes caused by Doxil was computed by the Wilcoxon matched-pairs signed rank test, (confidence interval 95%), or Mann-Whitney test (confidence interval 95%), as indicated. Differences between groups were considered significant at $P < 0.05$. Data are reported as median and interquartile range.

In vitro complement assay

Human serum samples from healthy volunteer donors, obtained through an institutionally approved phlebotomy protocol, were stored at -70°C until use. The ELISA-based method for quantification of serum S-protein-bound C terminal complex (SC5b-9) and levels of the catalytic subunit of Complement factor B (Bb) was performed as described earlier.^{49,50} In brief, the test polymers, at the concentration determined to be the IC50 value, and control compounds were incubated with different human sera for 45 min at 37°C in a shaking water bath (shaking rate of 80 rpm). After terminating the reaction by adding chilled specimen diluent (provided in the ELISA kits) at a 20-fold volume, samples were tested for SC5b-9 and Bb levels using the respective ELISA kits (TCC and Bb kits, Quidel Co, San Diego, CA), following the manufacturer's instructions. All reactions were tested in duplicates.

Results

In vivo complement activation by Doxil

To test complement-activating properties of Doxil, 12 pigs were administered 0.01 mg/kg Doxil, the equivalent of 0.06385 mg phospholipid per kg bodyweight (0.06385 mg PL/kg), as first injection. This represents minute amounts of Doxil, far below the doses clinically administered to patients, which underscores the importance of the anaphylactic reactions observed during these experiments.

Hemodynamic parameters were continuously monitored and recorded. We were paying focused attention to typical physiological changes characteristic to complement activation related pseudo-anaphylactic reaction, which include increase of pulmonary arterial pressure, increase or decrease of systemic arterial blood pressure, increase or decrease of heart rate, compromised cardiac output, dyspnea, apnea, increased or decreased end tidal CO₂, decreased arterial oxygen saturation measured by pulse oximetry, skin mottling, rash, etc. (Figure 8 and Figure 9)

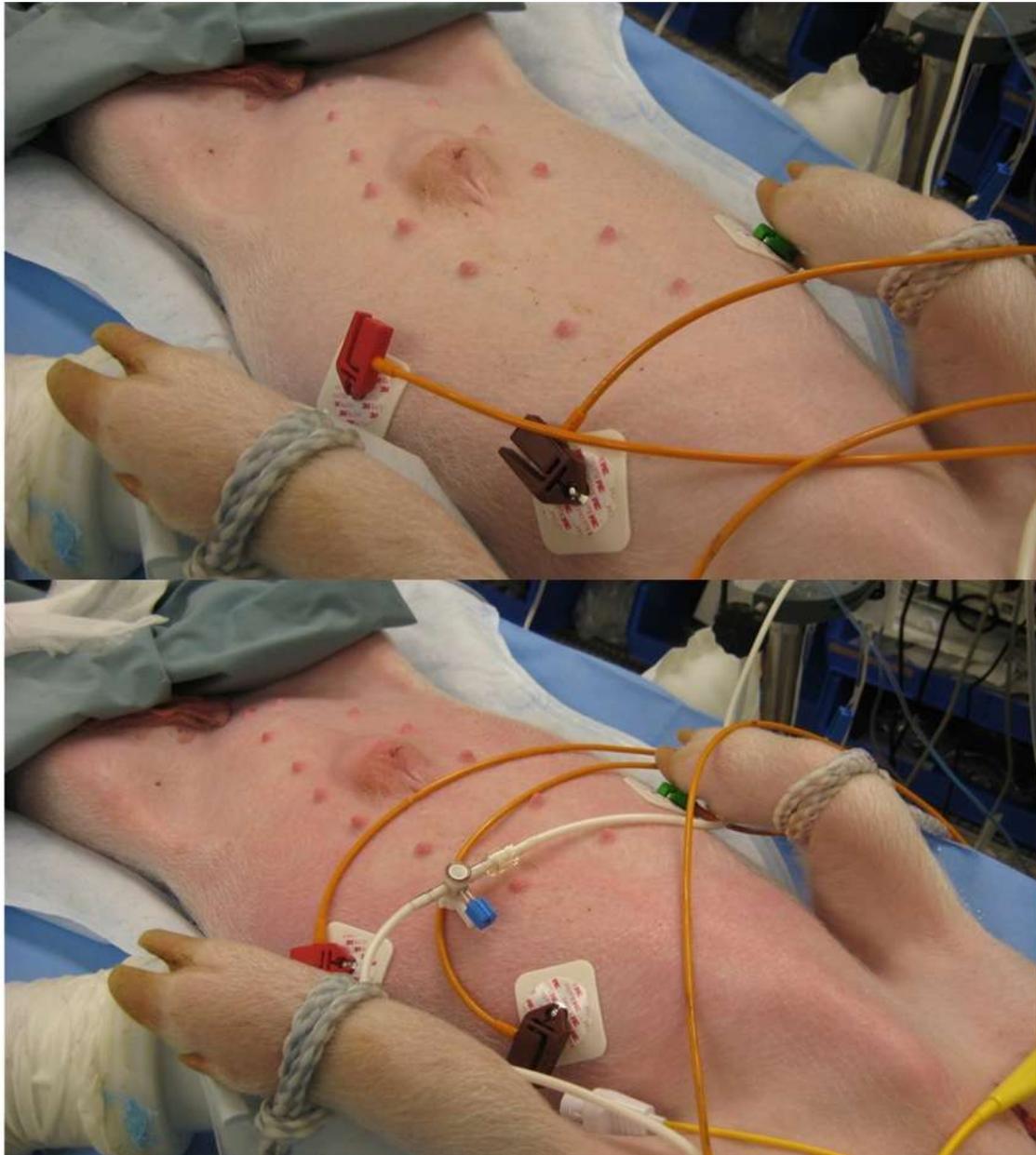


Figure 9: Skin reaction during CARPA. Above: normal skin before injection Below: rash, erythema, following injection of 0.06385 mg PL/kg Doxil. Cutaneous symptoms occur during severe reactions, with near lethal drop in systemic blood pressure, substantial increase in pulmonary arterial pressure, and often apnea.

These symptoms vary depending on the severity of the reaction. The most consistent sign of anaphylaxis in the pig model is acute pulmonary hypertension. In mild cases of CARPA the systemic arterial pressure increases or slightly decreases. However, during severe reactions, due to compromised cardiac output and/or peripheral vasodilation, the mean arterial pressure can drop to very low levels, occasionally even below the minimum required for the perfusion of vital organs. This is also reflected in the end tidal CO₂. On one hand high end tidal CO₂ could mean hypoventilation. However, if the cardiac output is insufficient to maintain proper perfusion of the lungs to enable gas exchange, the end tidal CO₂ drops. Heart rate can also change in both directions. During minor challenge heart rate slightly increases. However, during serious CARPA events paradoxical bradycardia can be observed. Described in detail in a previous paper by our group, this has been shown to be mediated by adenosine release from the ischemic heart.⁵² Also, if the animal can recover from a state of severe cardiovascular compromise with low cardiac output and blood pressure, usually a high amount of catecholamine is released into the circulation resulting in a “rebound” tachycardia with high blood pressure.

The physiological changes observed in the pig model also correspond to clinical symptoms of CARPA: dyspnea, light-headedness, chest pain, etc.

After the injection of Doxil, we observed acute pulmonary hypertension in all animals, within a few minutes following the injection. The mean pulmonary arterial pressure (PAP) increased significantly ($p=0.0005$, $n=12$) from a median of 16.5 (15-20.25) mmHg to 42 (34.5-48) mmHg. (Figure 10)

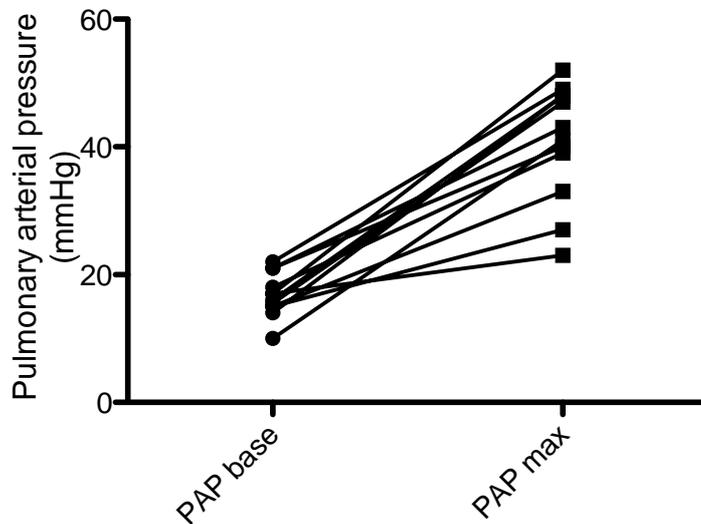


Figure 10. PAP changes following 1st injection of Doxil (0.06385 mg PL/kg)

Each pair of connected points represents one animal, and pre-injection baseline and post-injection maximum pressure values are shown. $p=0.0005$, $n=12$, PAP baseline 16.5 (15-20.25) mmHg, PAP maximum change 42 (34.5-48) mmHg

In our model, the symptom of CAPRA can be an increase or decrease of systemic mean arterial pressure. Because the Wilcoxon matched-pairs signed rank test used for analysis of our data compares the medians of two paired groups, it is possible that individual variations among the animals cancel out each other during analysis. As a result, the baseline and post-injection median of the SAP values (99 and 106 mmHg, respectively) was not significantly different ($p=0.2061$), because in some cases the pressure increased, in other cases it decreased. However, there were some animals where the blood pressure dropped to life-threateningly low levels. Notably, we observed changes from 111 to 32 mmHg, from 83 to 30 mmHg, from 63 to 31 mmHg, from 98 to 52 mmHg (Figure 11)

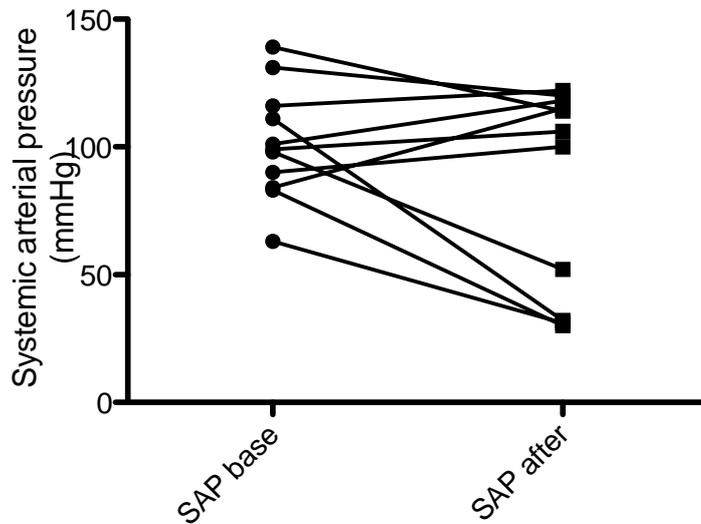


Figure 11. SAP changes following 1st injection of Doxil (0.06385 mg PL/kg)
 $p=0.2061$, $n=11$, SAP baseline 99 (84-116) mmHg, SAP after injection 106 (32-118) mmHg

The dynamics of a typical reaction in one of the animals is illustrated in Figure 12. The pulmonary arterial pressure (PAP) increased from a baseline of 16 to a maximum of 48 mmHg. This was followed by a decrease in systemic arterial pressure (SAP) from a baseline of 111 mmHg to a minimum of 32 mmHg. The drop in SAP is also accompanied by paradoxical bradycardia, i.e. at the time of the blood pressure reaching 31 mmHg, representing life-threatening hypotension, instead of compensatory tachycardia we see a temporary decrease in heart rate. This is likely mediated by adenosine release from the ischemic myocardium.

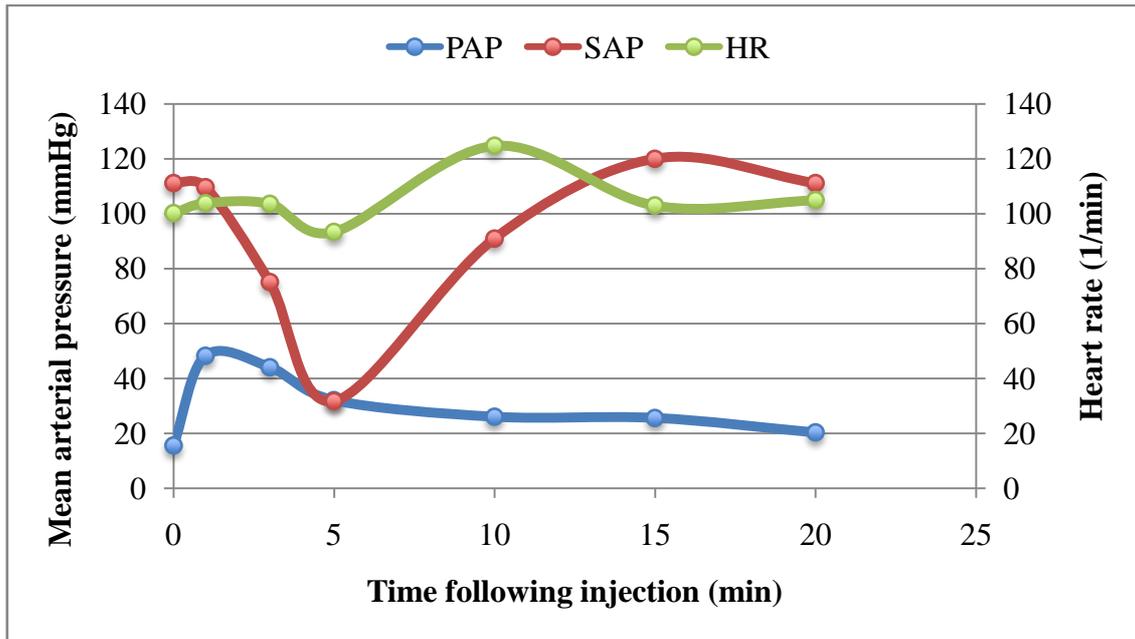


Figure 12. Typical hemodynamic changes following injection of Doxil. The representative data is from an experiment following the administration of 0.06385 mg PL/kg Doxil at time 0 min on the graph.

These changes resembled typical CARPA reactions, and were comparable to the reactions observed following the administration of Zymosan, a potent known complement activator used as positive control.

Summary:

First injection of Doxil causes clinically significant acute pulmonary arterial hypertension.

We compared baseline and maximum of PAP after 1st Doxil injection in the control group using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%.

PAP increased from a median of 16.5 mmHg to 42 mmHg ($p=0.0005$, $n=12$)

SAP change was statistically and clinically not significant (99 to 106 mmHg), except for some cases.

Tolerance after first Doxil injection

To test whether the first reaction can be repeated with subsequent doses, in seven animals the first Doxil injection was followed by a second identical dose of Doxil injection (0.06385 mg PL/kg). Although the change in PAP from a median of 17 (14-19) mmHg to 19 (16-20) mmHg was statistically significant ($p=0.0177$, $n=7$), such a slight increase is clinically irrelevant, and hence we don't consider it to be a serious anaphylactic reaction. The systemic arterial pressure did not change (88.5 (74-95.5)mmHg at baseline and 89 (80.25-98.75) mmHg following injection).

Also, as it can be seen in Figure 13 below, that following the second injection of Doxil there were no individual animals that suffered acute pulmonary hypertension or a drop in systemic blood pressure.

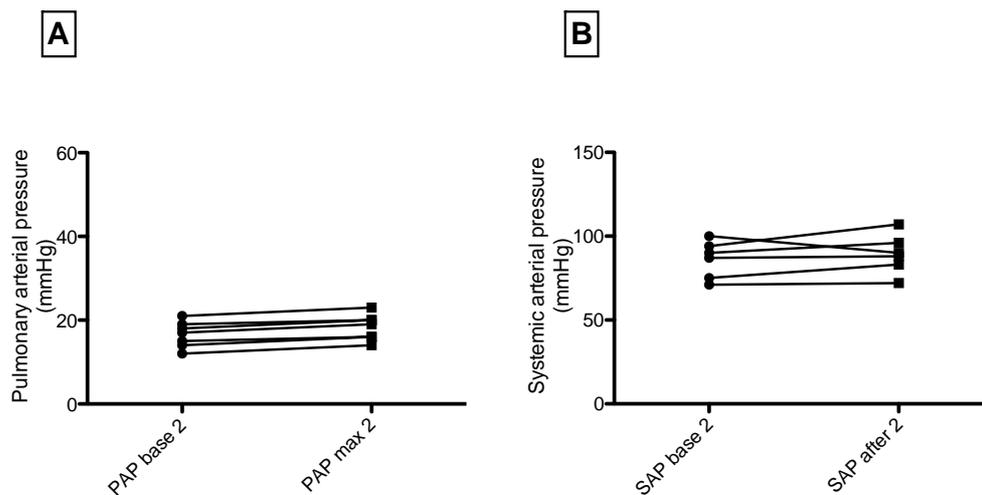


Figure 13. (A) PAP and (B) SAP changes following the 2nd injection of Doxil.

Each point represents one animal, and pre- and post-injection pressure values for the same animal are connected with a line. It's apparent that in contrast to the first injection, there are no steep lines representing a major increase or decrease in the blood pressures of any of the subjects. (A) PAP: $p=0.0177$, $n=7$, baseline 17 (14-19) mmHg, maximum change 19 (16-20) mmHg; (B) SAP: $p=0.2932$, $n=6$, baseline 88.5 (74-95.5) mmHg, maximum change 89 (80.25-98.75) mmHg

To test whether tolerance can be breached with a higher dose, six animals received an additional five-fold bolus dose of Doxil (0.31925 mg PL/kg) as a third injection. No statistical difference could be shown between pre- and post-injection pressures either in the pulmonary arterial pressure (17 (12.75-19.5) and 20 (17.75-22.25) mmHg, respectively), or in the systemic arterial pressures (89.5 (77.75-97.75) and 86 (74.5-99.75) mmHg, respectively). (Figure 14)

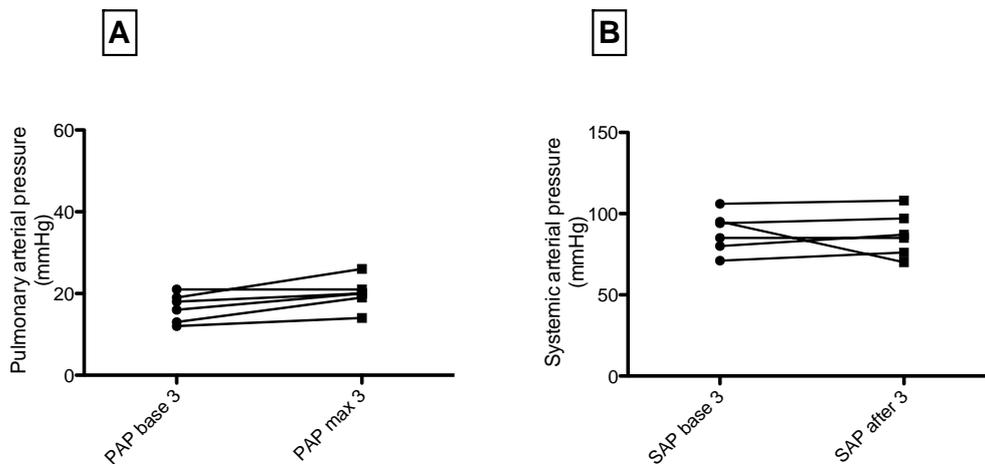


Figure 14.(A) PAP and (B) SAP changes following third injection of Doxil with 5x dose (0.31925 mg PL/kg)

There were no significant changes, even at the individual level. Tolerance was shown to 5x dose. (A) PAP: $p=0.0579$, $n=6$, baseline 17 (12.75-19.5) mmHg, maximum change 20 (17.75-22.25) mmHg; (B) SAP: $p=0.625$, $n=6$, baseline 89.5 (77.75-97.75) mmHg, maximum change 86 (74.5-99.75) mmHg.

Another compelling evidence for tachyphylaxis is that comparing the maximal PAP values in the animals that have received all 3 consecutive Doxil injections, we found significantly higher pulmonary pressure after the 1st than after the 2nd injection (44.5 (31.5-49.75) versus 17.5 (15.5-20.75) mmHg, respectively; $p=0.0355$), while the baselines were not different. This is illustrated in Figure 15. Comparison of the PAP maximum values between the 1st and 3rd injections showed similar difference (44.5 (31.5-49.75) vs. 20 (17.75-22.25) mmHg, respectively; $p=0.0355$).

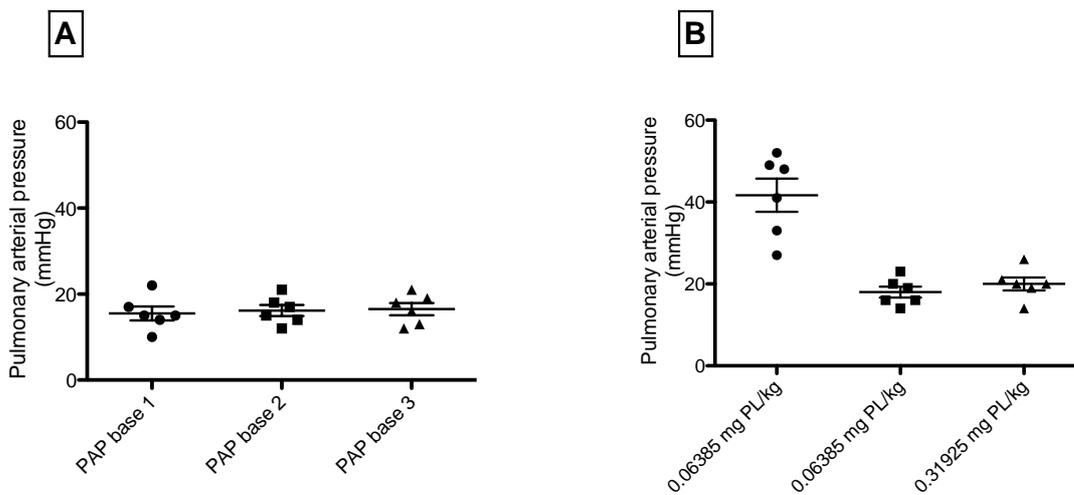


Figure 15. Comparison of (A) baselines and (B) post-injection maximums of pulmonary arterial pressure.

Baselines were not different ($p=0.2795$ for 1 vs. 2, and $p=0.2809$ for 1 vs. 3, $n=6$), while maximum change after 1st injection was higher than after the 2nd ($p=0.0355$) and 3rd ($p=0.0355$) injections.

To test whether tolerance is specific to Doxil, after the Doxil injections we administered two sequential doses of 0.5 mg/kg Zymosan, a known complement activator as positive control. The first injection caused a significant increase in PAP from 17 (14-18) mmHg to 46 (30-50) mmHg ($p=0.0156$, $n=7$) suggesting that the tolerance to Doxil is specific, and reactivity to Zymosan is preserved. The PAP also changed after the second injection of an identical dose of 0.5 mg/kg Zymosan from 21 (17-24.25) mmHg to 50.5 (42.25-57.25) mmHg, ($p=0.0355$, $n=6$), suggesting that Zymosan does not induce self-

tolerance under these circumstances. (Figure 16)

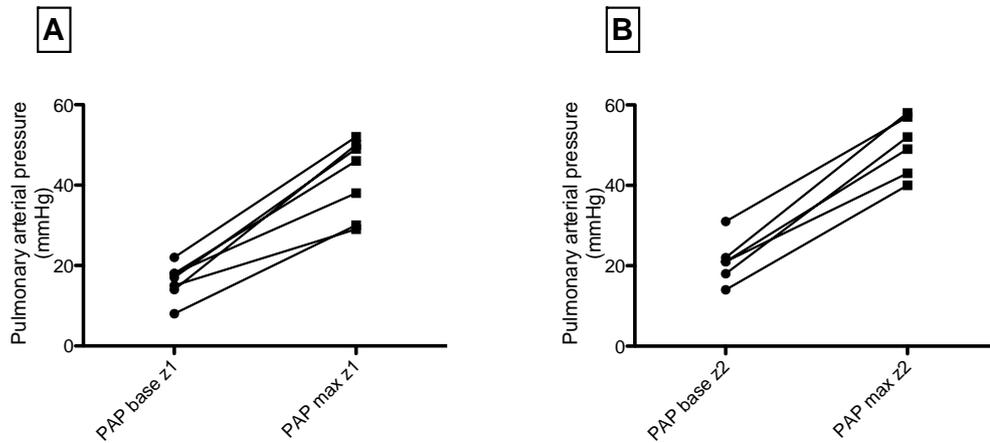


Figure 16. PAP changes following (A) first and (B) second injection of Zymosan (0.5 mg/kg) after Doxil injections.

PAP increased from 17 to 46 mmHg after the 1st injection ($p=0.0156$, $n=7$) and from 21 to 50.05 mmHg ($p=0.0355$, $n=6$) after the 2nd injection.

Summary:

There is tachyphylaxis for subsequent Doxil injections and they do not cause clinically significant acute pulmonary arterial hypertension or changes in systemic blood pressure.

a) We compared baseline and maximum of PAP after 2nd Doxil injection in control group using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%.

PAP increased from a median of 17 mmHg to 19 mmHg ($p=0.0177$, $n=7$). This is statistically significant, but biologically not relevant.

b) We compared baseline and maximum of PAP after 3rd Doxil injection in control group using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%.

PAP before and after injection was 17 and 20 mmHg, respectively; the difference was not significant ($p=0.0579$, $n=6$)

c) We compared baseline PAP of 1st and baseline PAP of 2nd Doxil injection, and

then compared maximum PAP after 1st and maximum PAP after 2nd Doxil injection in the control group using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%.

Maximum of PAP was higher after 1st than after 2nd Doxil injection ($p=0.0355$, median of 44.5 mmHg vs. 17.5 mmHg, respectively). Baseline PAP was not different between 1st and 2nd Doxil injections ($p=0.2795$).

Comparison of 1st and 3rd injections showed higher maximum PAP after 1st injection ($p=0.0355$, 44.5 vs. 20 mmHg) and baselines were not different ($p=0.2809$).

d) We compared baseline and maximum of PAP after the 1st injection of Zymosan following the Doxil injections using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%. We repeated the same analysis for the 2nd injection of Zymosan

PAP increased from a median of 16.5 mmHg to 42 mmHg ($p=0.0313$ $n=6$) following the 1st Zymosan injection and from a median of 21 mmHg to 49 mmHg ($p=0.0625$, $n=5$) after the 2nd Zymosan dose.

The first injection of Doxil caused acute pulmonary hypertension, but the repeated injection of the same dose did not increase the pulmonary arterial pressure, nor did the third five-fold dose, while the 1st and 2nd injection of positive control Zymosan still provoked CARPA reaction. (Figure 17)

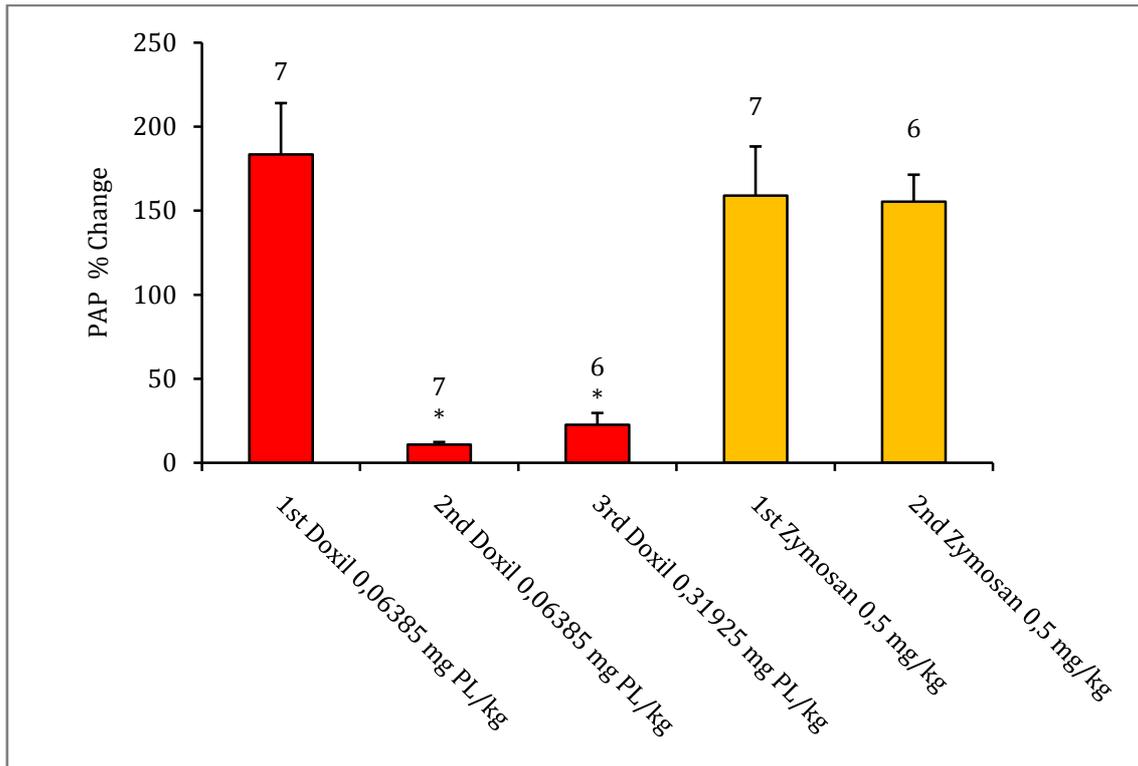


Figure 17. Tachyphylaxis following first injection of Doxil

Changes of pulmonary arterial pressure are shown as percentage increase of PAP following the injection (Data is shown as mean + SEM; 0 means no change). Number of animals from the series that received the injections is shown above each column. * Marks significant difference compared to the change after 1st Doxil injection ($p=0.0156$ 1st versus 2nd; $p=0.0313$ 1st vs. 3rd injection). The changes in pulmonary arterial pressure after the 1st and 2nd Zymosan injections were not different from changes after 1st injection of Doxil.

Utilizing tachyphylaxis to prevent severe CARPA reaction

To study whether the tachyphylaxis phenomenon can be used to prevent severe CARPA reaction provoked by Doxil injections, we treated five animals with a relatively slow infusion of Doxebo, which is a liposome preparation with the same chemical composition as Doxil, with the sole difference that it does not encapsulate doxorubicin.

Doxebo preparation containing 0.06385 mg PL/kg was infused over 15-30 minutes and hemodynamic parameters were monitored. PAP didn't change significantly ($p=0.25$, $n=5$), and the SAP didn't change either ($p=0.7865$, $n=5$). (Figure 18)

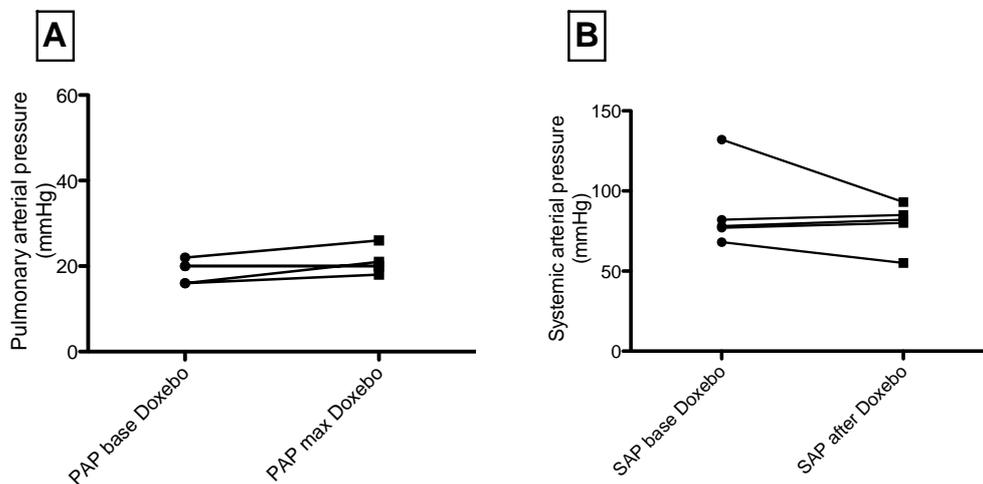


Figure 18. Hemodynamic changes during and following infusion of Doxebo (0.06385 mg PL/kg)

(A) PAP did not change ($p=0.25$, $n=5$); and (B) SAP did not change ($p=0.7865$, $n=5$) during and after Doxebo pretreatment.

Shortly after the Doxebo infusion a 0.031925 mg PL/kg dose of Doxil bolus was injected. As a result, the PAP didn't change significantly ($p=0.4164$, $n=5$) and nor did the SAP ($p=1$, $n=5$). (Figure 19)

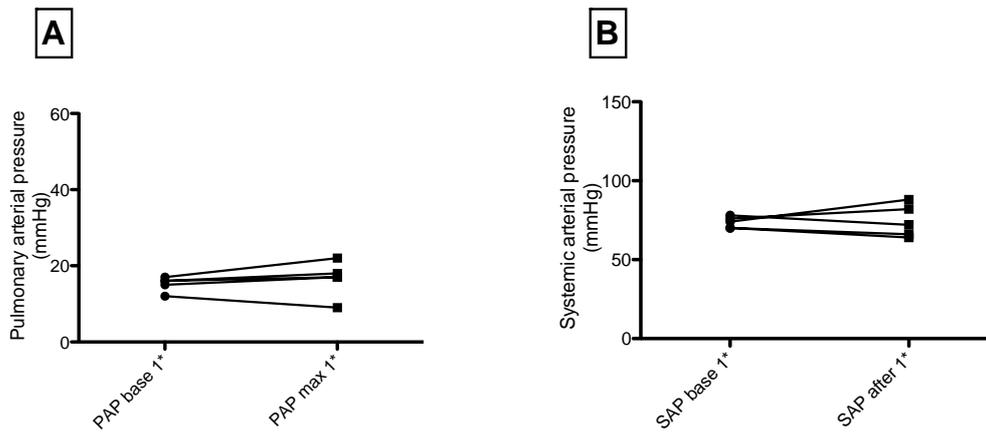


Figure 19. Hemodynamic changes following 1st injection of Doxil (0.031925 mg PL/kg) after Doxebo pretreatment

(A) PAP remained unchanged ($p=0.4164$, $n=5$) and so did the (B) SAP ($p=1$, $n=5$) after the 1st injection of Doxil following tolerization with Doxebo.

After the first Doxil a second, larger dose of Doxil was injected, varying between 0.06385 and 0.31925 mg PL/kg, but the tolerance could not have been breached, and neither the PAP, nor the SAP did not change significantly ($p=0.625$ and $p=0.7865$, respectively, $n=5$). (Figure 20)

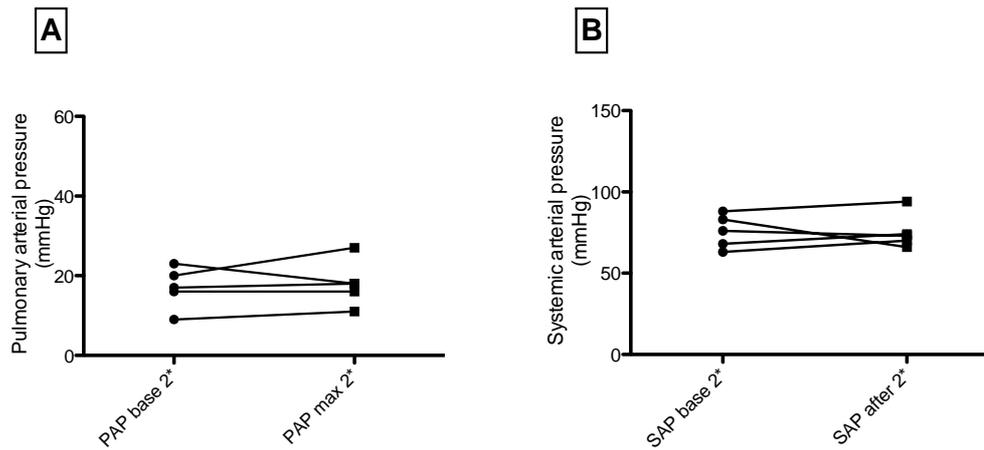


Figure 20. Hemodynamic changes following 2nd injection of Doxil (0.06385-0.31925 mg PL/kg) after Doxebo pretreatment

Neither the (A) PAP, nor the (B) SAP did not change significantly ($p= 0.625$ and $p=0.7865$, respectively, $n=5$) following the second dose of Doxil.

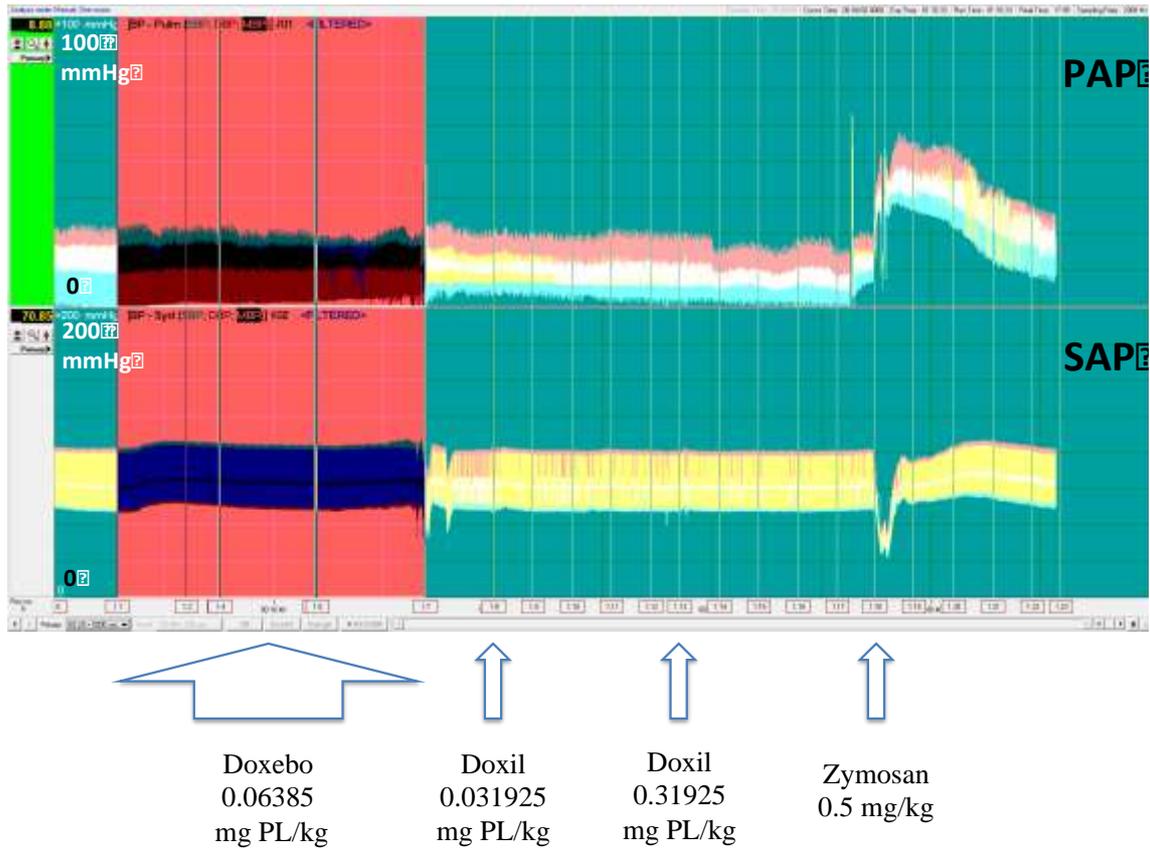


Figure 21. A screenshot from a representative recording of a Doxebotolerization experiment.

Arrows mark the time of infusion or injection of the various substances. The duration of the Doxebo infusion is highlighted. The scale of the PAP curve is 0-100 mmHg, the scale of the SAP curve is 0-200 mmHg.

In Figure 21 it is apparent that there was no CARPA reaction to either the Doxebo, or the subsequent Doxil injections (marked by arrows). However, the positive control Zymosan still provoked acute pulmonary hypertension and a transient, but severe drop in systemic arterial pressure.

In contrast, the next figure (Figure 22) shows a recording of an experiment without Doxebo pretreatment. The first bolus of Doxil provoked acute pulmonary hypertension; so severe that it was immediately followed by the almost complete collapse of circulation (see the drop in the systemic blood pressure to nearly 0 mmHg). When the released catecholamines could finally circulate and exert their effect, there was a rebound hypertension, and the temporarily normalized pulmonary arterial pressure reached even higher levels. The subsequent Doxil boluses did not provoke circulatory reactions, but the positive control Zymosan's effects were again very similar to those observed after the first injection of Doxil.

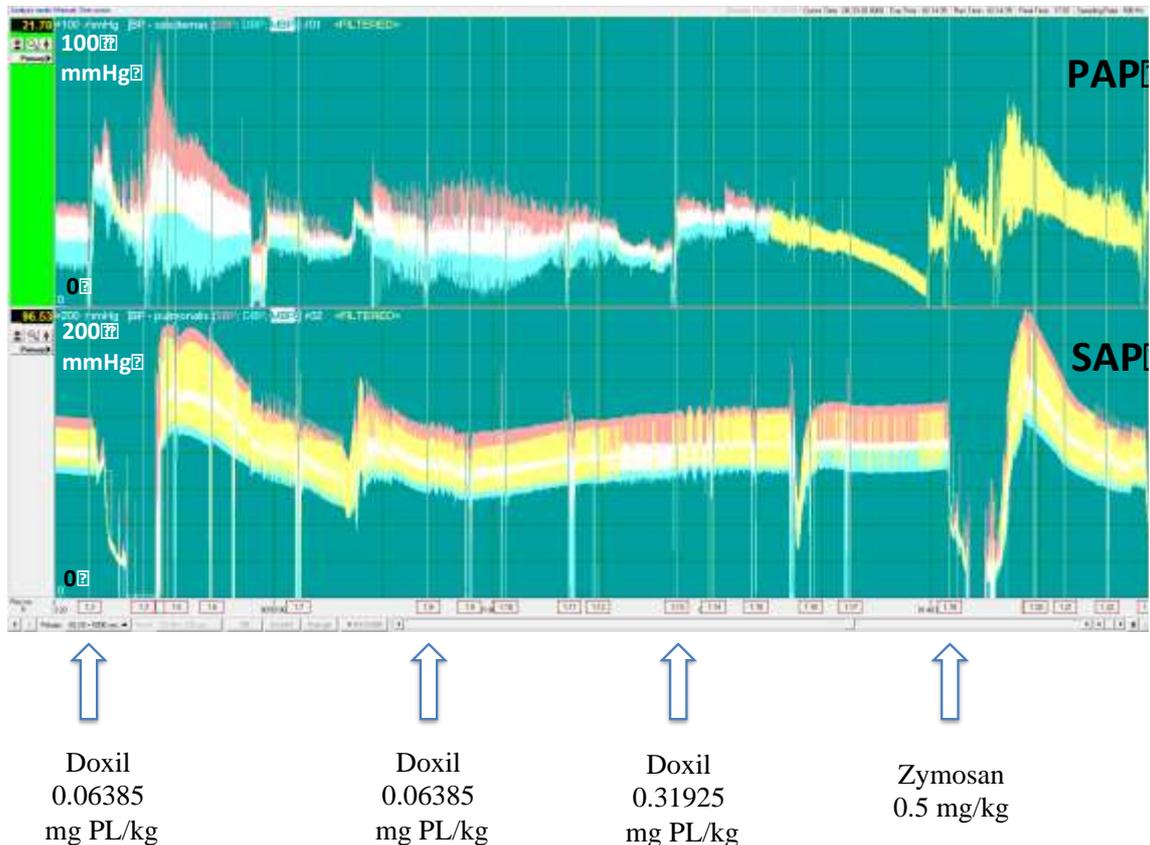


Figure 22. Screenshot of a typical recording of PAP and SAP traces during sequential injections of Doxil and Zymosan.

Looking at the hemodynamic changes following the injection of the positive control Zymosan in the group of animals without Doxebo pretreatment (Figure 22) we found that the ability to develop acute pulmonary hypertension remained intact; hence the tachyphylaxis was specific to Doxil. However, although the median of PAP values in the Doxebo-pretreated treatment group before and after the injection of Zymosan were 16(95% confidence interval (CI) 11-20.5) and 46(95% CI 25.38-63.62) mmHg, respectively, and the increase in pulmonary arterial pressure was at least 140% in all the animals, the analysis with two-tailed Wilcoxon matched-pairs signed rank test did not show statistical significance ($p=0.125$), probably due to the low number of samples ($n=4$). (Figure 23)

Summary:

a) First administration of Doxebo (placebo Doxil) does not cause clinically significant acute pulmonary arterial hypertension.

We compared the baseline pulmonary arterial pressure before and the maximum of pulmonary arterial pressure during and after Doxebo infusion in treatment group using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%

PAP remained unchanged. ($p=0.25$, median of 20 mmHg, $n=5$)

b) Preliminary Doxebo infusion prevents acute pulmonary hypertension caused by subsequent Doxil injections.

We compared the baseline and the maximum of pulmonary arterial pressure after Doxil injection in the treatment group using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%

PAP remained unchanged both after the low and high doses of Doxil. ($p=0.4164$, $n=5$, and $p=0.625$, $n=5$, respectively)

c) Zymosan used as positive control following Doxil injections without Doxebo pretreatment provoked acute pulmonary hypertension. This reaction may not be affected by pretreatment with Doxebo, however the results are not statistically significant. Tolerance induction by Doxebo and Doxil is specific.

We compared the baseline and the maximum of PAP after Zymosan injection in the Doxebo pretreatment group using Wilcoxon matched-pairs signed rank test, with confidence interval of 95%.

PAP increase was not statistically significant in the Doxebo pretreatment group.

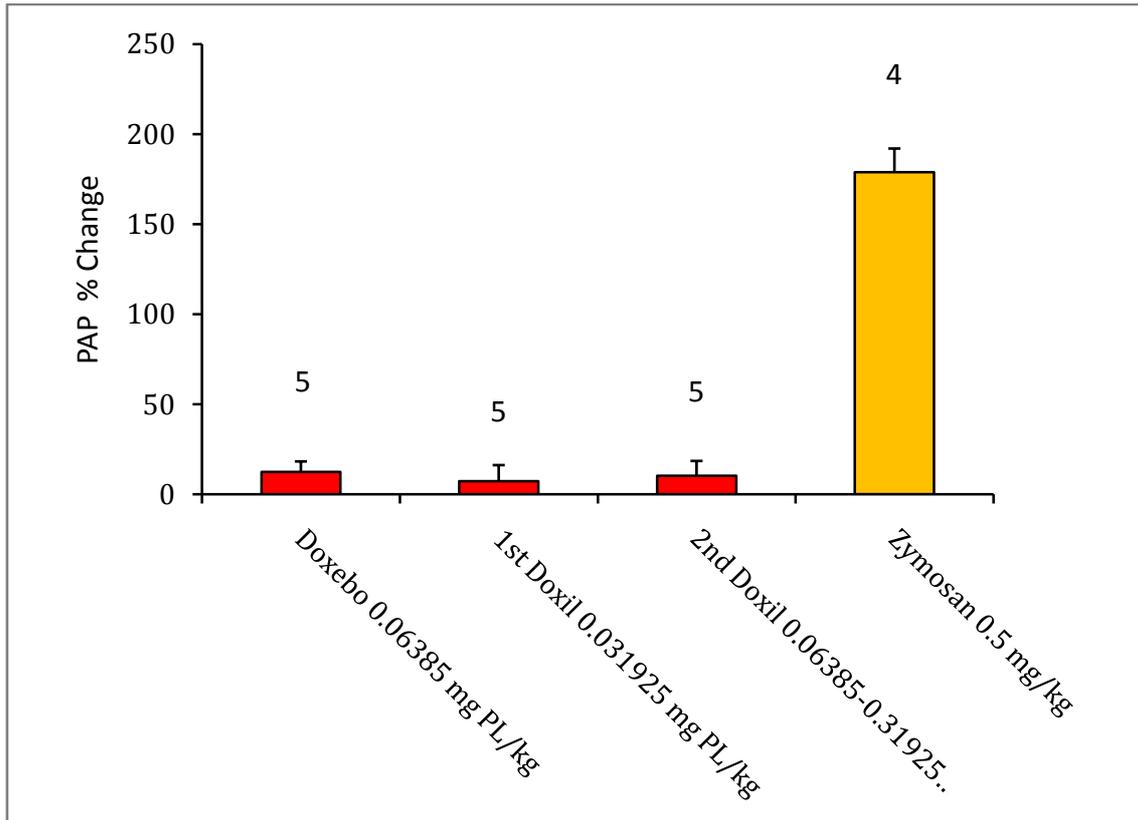


Figure 23. Tolerance after Doxebo infusion

Slow infusion of Doxebo, without causing acute pulmonary hypertension, prevented the reactions to the subsequent Doxil injections. Changes of pulmonary arterial pressure are shown as maximum of PAP following the injection expressed in percentage of baseline. (Data is shown as mean + SEM) Number of animals from the series that received the injections is shown above each column.

Other examples of the utility of the in vivo model

Predicting complement activation by polymers

In vitro complement activation tests show high inter-individual variation because the sera used for each measurement have different sensitivity. In comparison, 100% of the pigs are sensitive and exhibit complement activation related pseudo-anaphylactic reaction to activating substances. In some cases when in vitro tests yield weak, or potentially false negative results, confirmation by in vivo tests can reveal complement-activating properties of the drug. This is illustrated by our series of experiments testing polyethylene imine (PEI) preparations.

In vitro measurements

To examine the in vitro complement activation by the PEI polymers, nineteen series of SC5b-9 ELISA measurements were performed in 6 different human sera. 80 µg/ml PEI 25kDa caused significant elevation of SC5b-9 in 3 out of the 9 sera, while the other substances did not exhibit C activation (Figure 24 B-D). In the other 6 sera PEI 25kDa was not reactogenic, although the positive control Zymosan produced increased SC5b-9 levels. Also, the increase in SC5b-9 in the reactive sera varied from 2 to 10-fold, and in one serum that was sensitive, the increase in SC5b-9 to the PEI 25kDa stimulus appeared to be dose dependent (Figure 24 E). These results suggest that there is high inconsistency among the in vitro test results, and there is substantial individual variation in sensitivity. In this case, 66% of the time the tests were negative for a compound that otherwise activates complement.

It's also worth mentioning, that in 1 of the 6 sera PEI 5kDa was reactogenic at a 30-fold higher dose than PEI 25kDa (2.5 mg/kg, data not shown), indicating that PEI 5kDa has the potential to activate complement.

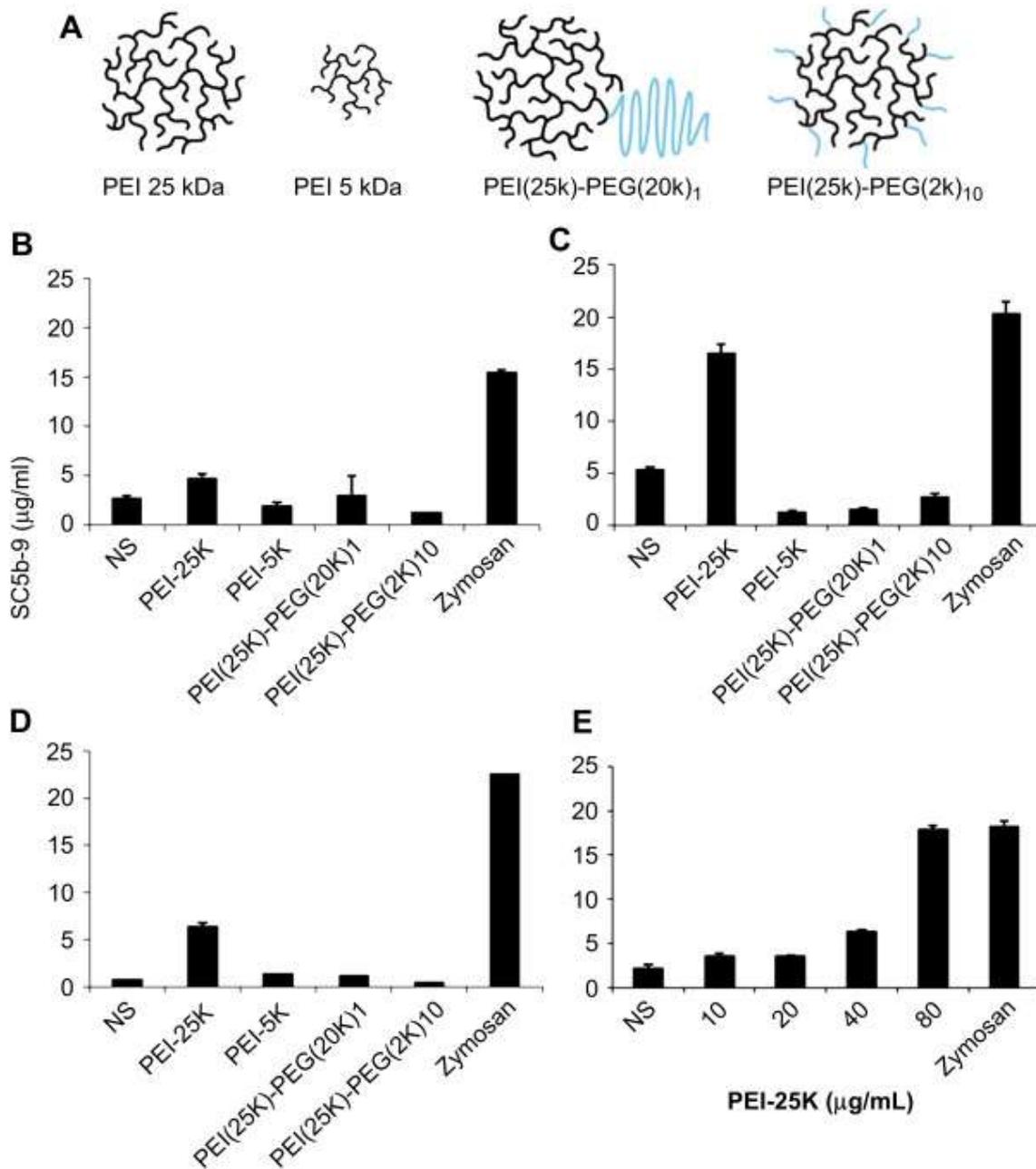


Figure 24. (A) Schematic composition of the polymers tested. (B)-(E) In vitro complement activation by the IC₅₀ concentrations of the above polymers clearly differed depending on the serum. Unmodified PEI 25kDa, however, elicited the highest activity in all sera while decrease in molecular weight and PEGylation caused a decrease in immunogenicity.

Combining the results from all the nineteen series of measurements, we found significant complement activation only by PEI 25kDa when comparing the SC5b-9 concentration in the serum activated with the test substances expressed as a percentage of the SC5b-9 concentration in the serum from the same donor activated with Zymosan ($p=0.0273$ NS vs. PEI 25kDa). Activation with all the other substances was not different from normal saline. (Figure 25)

Concentration of SC5b-9 expressed as % of activation with Zymosan

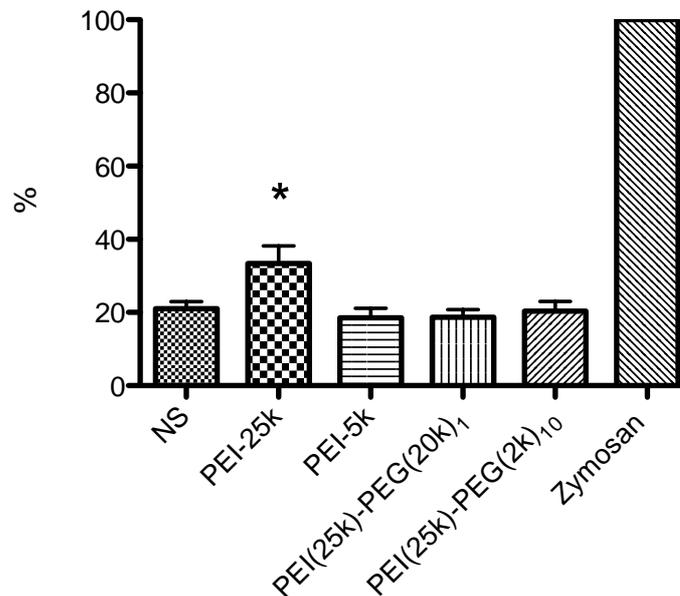


Figure 25. In vitro complement activation by the various polymers as measure by the SC5b-9 assay and expressed in percentage of activation by the positive control Zymosan.

In vivo tests

To explore the in vivo complement-activating properties of the polymers, 13 pigs were injected with the preparations and hemodynamic changes were analyzed.

All of the tested polymers were active in terms of causing significant hemodynamic changes, including a rise of PAP, rise or decline of SAP, change of heart rate, and ECG alterations. In some cases the reaction was practically lethal and required active resuscitation efforts to save the animal.

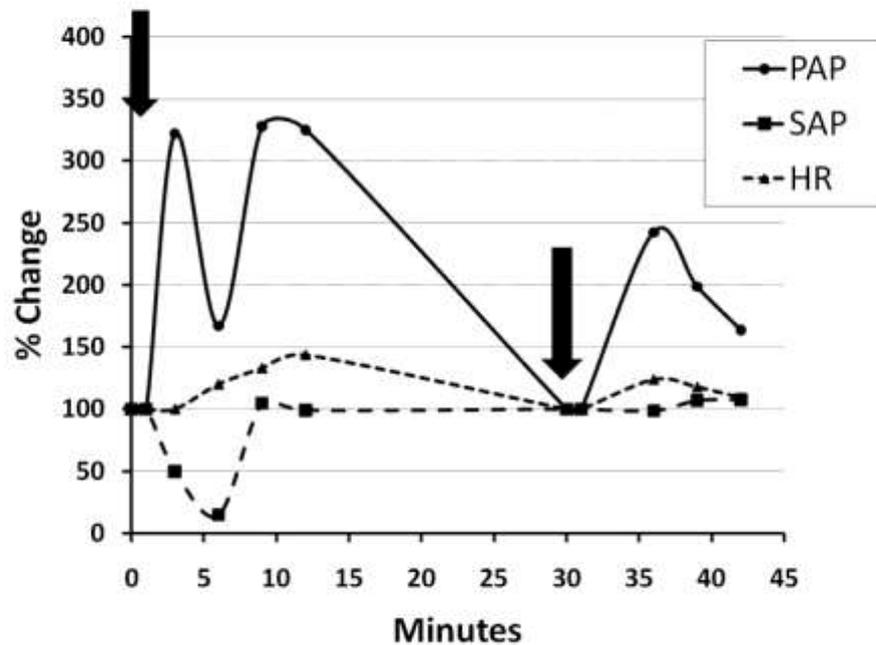


Figure 26. As a demonstration of the complement activating potential of the polymers, hemodynamic reactions (expressed as % of the baseline) following of two injections of 0.1 mg/kg PEI 25kDa (marked with arrows) are shown. The more than threefold increase in pulmonary pressure after the first injection is coupled with a drop in the systemic blood pressure to almost 0, representing a typical life-threatening CARPA reaction. The temporary decrease in PAP can be explained by severe cardiovascular compromise and almost complete collapse of circulation. After recovery from the first challenge, the 2nd identical i.v. dose of PEI 25kDa also provoked hemodynamic reactions in the form of a 2.5 fold increase in PAP. This excludes the presence of complete tachyphylaxis, although the reaction is less severe compared to the first one, implying a possibility of some desensitization.

Because of the low number of animals per group, it is not possible to perform a meaningful statistical analysis of the hemodynamic changes provoked by each of the different polymers separately. For this reason, all the individual experiments are presented in the next figure (Figure 27).

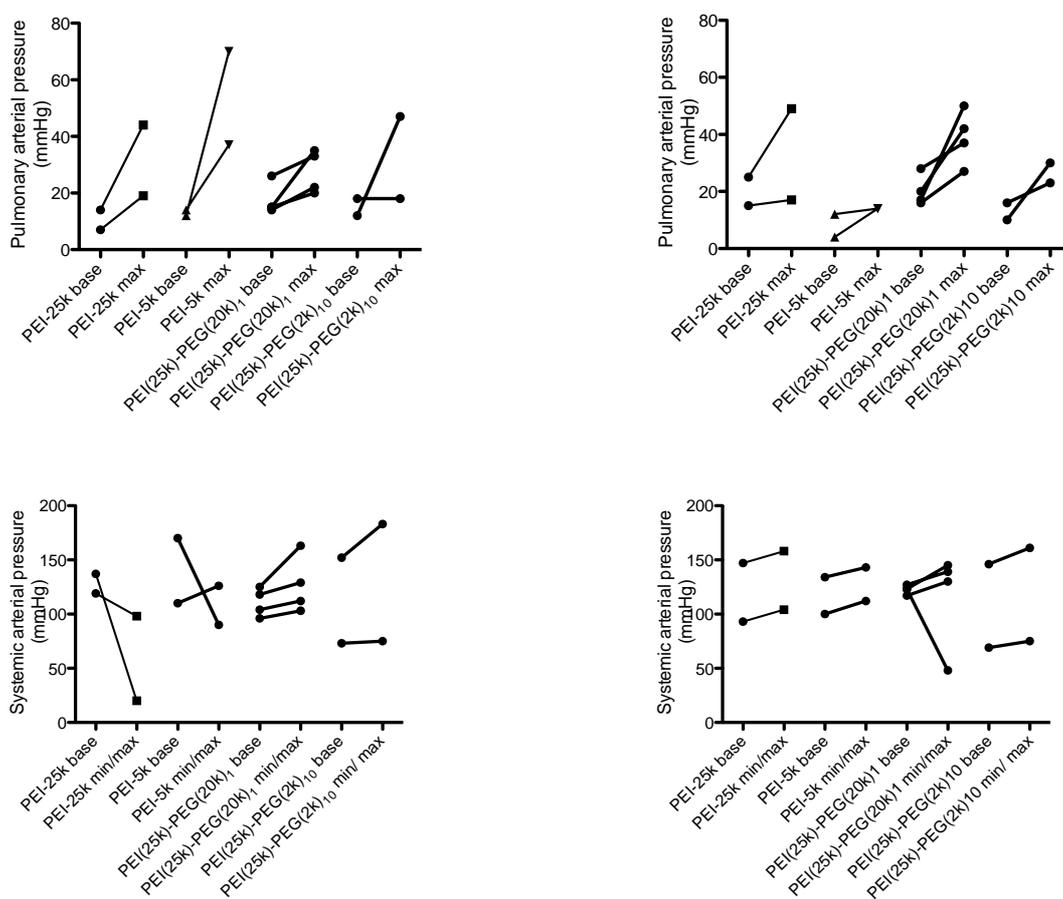


Figure 27. Change in pulmonary mean arterial pressure and systemic mean arterial pressure following injection of 0.1 mg/kg bolus of polymers. Results for the first injection are on the left, data for the second injection are on the right. (n=2 for PEI 25kDa, PEI 5kDa and PEI(25k)-PEG(2k)₁₀ ; n=4 for PEI(25k)-PEG(20k)₁)

It is evident that in a number of cases, the pulmonary arterial pressure severely increased after the 1st injection, as well as after the 2nd injection of the same dose of the same polymers. In some cases even serious systemic hypotension developed.

Taking all the animals into account, the pulmonary arterial pressure significantly increased after the first injection from a median of 14 (12-15.75) to 34 (19.75-44.75) mmHg ($p=0.0039$, $n=10$). Nonetheless, the increase after the second injections was also significant from 16 (11.5-21.25) to 28.5 (16.25-43.75) mmHg, ($p=0.0059$, $n=10$) and there was no difference between the changes of PAP after the 1st and 2nd injection (148.8% (31.73-233.6) vs. 82% (28.27-195.6) increase, respectively; $p=0.2324$, $n=10$). (Figure 28) This means that in general, we did not observe tachyphylaxis during the duplicate injection of the substances, although it is possible that there are differences between the polymers in this regard.

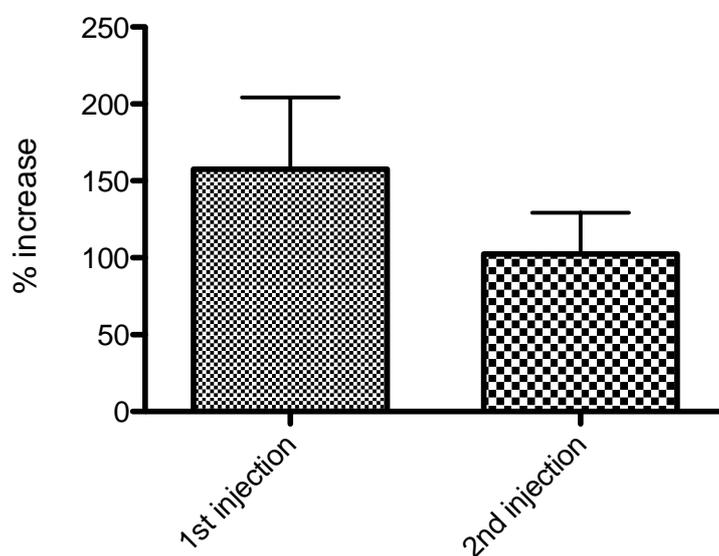


Figure 28. Increase in pulmonary pressure following duplicate injections of polymers. The doses were identical, 0.1mg/kg. Although the increase in PAP seems to be smaller after the second injection, implying some desensitization, the difference between the changes was not significant. ($n=10$)

Summary:

a) Acute pulmonary hypertension developed after the first injections of polymers.

We compared the baseline and the maximum of PAP after the 1st polymer injections in all subjects using Wilcoxon matched-pairs signed rank test, with a confidence interval

of 95%.

PAP increased from a median of 14 mmHg to 34 mmHg ($p=0.0039$, $n=10$). SAP did not change.

b) Acute pulmonary hypertension developed after the second injections of polymers.

We compared the baseline and the maximum of PAP after the 2nd polymer injections in all subjects using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%.

PAP increased from a median of 16 mmHg to 28.5 mmHg ($p=0.0059$, $n=10$). SAP did not change.

c) There was no difference between the changes of PAP after the 1st and the 2nd injection.

We compared changes of PAP after 1st and 2nd injections using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%.

We found no significant difference. ($p=0.2324$)

Alternative species for CARPA testing

To assess the utility of dogs as an in vivo animal model for testing complement activation by nanomedicines, we studied their hemodynamic reactions to the intravenously administered liposomal drug Ambisome.

Six mongrel dogs were anesthetized and injected with the preparation following the same experimental design as used for the porcine Doxil experiments.

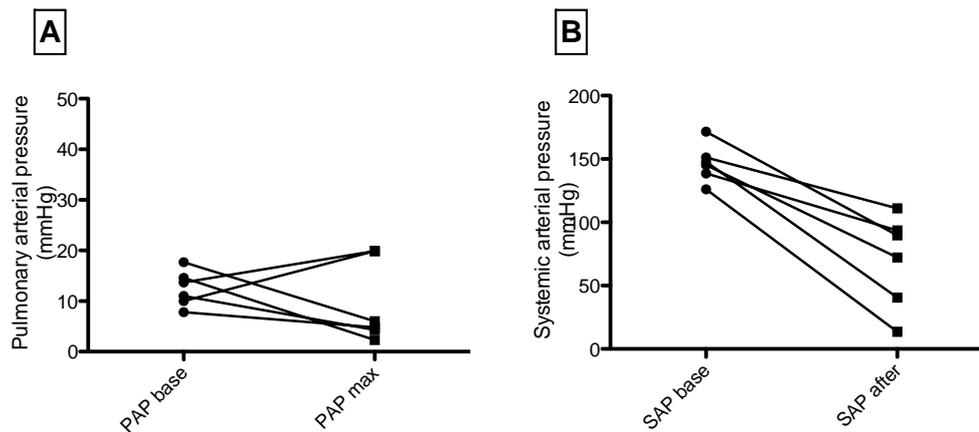


Figure 29.(A) PAP and (B) SAP changes following the 1st injection of Ambisome (0.01 mg/kg)

As it is apparent in Figure 29, the most consistent hemodynamic change in dogs following the injection of Ambisome was a drop in systemic blood pressure from 146.5 (135.4-156.4) mmHg to 81 (33.75-97.9) mmHg ($p=0.0313$, $n=6$). However, unlike during the pig experiments with liposomes, the pulmonary arterial pressure did not rise ($p=0.4375$, $n=6$).

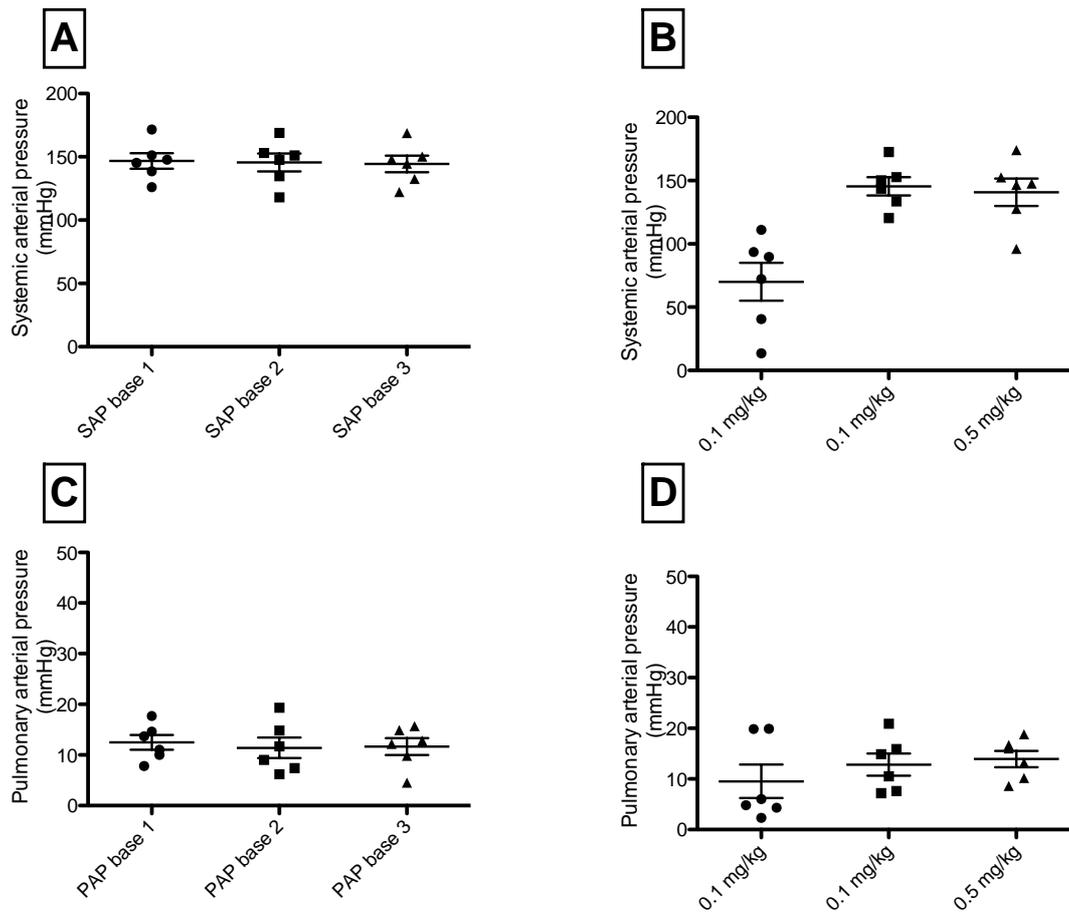


Figure 30. Tachyphylaxis following repeated administration of Ambisome in dog (A) Baseline systemic arterial pressures; (B) systemic arterial pressure following repeated doses of Ambisome; (C) baseline pulmonary arterial pressures; (D) pulmonary arterial pressures following repeated doses of Ambisome. n=6

Just like in the pig model, we observed tachyphylaxis during repeated administration of the liposomes. There was neither PAP nor SAP response to the second 0.1 mg/kg dose of Ambisome ($p=0.0625$ and $p=0.8438$, respectively, $n=6$), and the third five-fold bolus also proved to be non-reactogenic ($p=0.0938$ and $p=1$, PAP and SAP respectively, $n=6$) regarding the monitored hemodynamic parameters. (Figure 30)

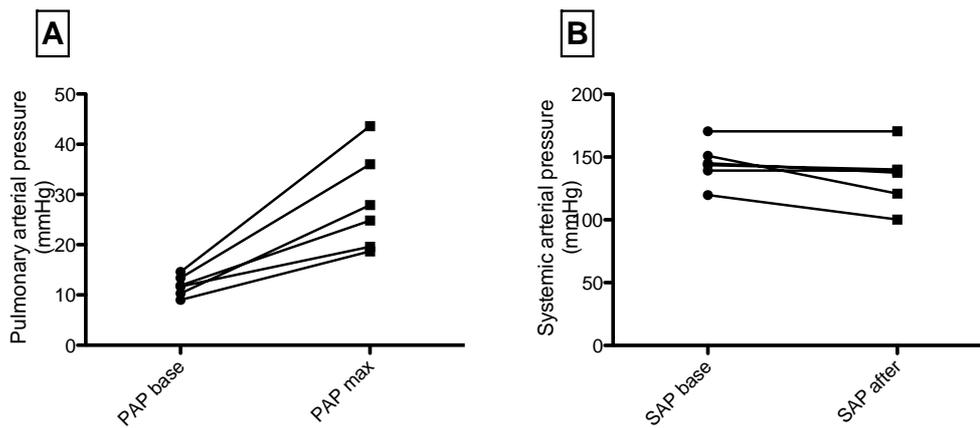


Figure 31. Changes in (A) pulmonary arterial pressure and (B) systemic arterial pressure following the administration of the positive control Zymosan 0.1 mg/kg (n=6)

Conversely, injection of 0.1 mg/kg Zymosan as positive control provoked significant increase in the pulmonary arterial pressure ($p=0.0313$, $n=6$) from a baseline of 11.8 (9.975-13.7) to 26.35 (19.38-37.9), while the systemic arterial pressure remained unchanged ($p=0.0625$, $n=6$). (Figure 31) The reason why Zymosan and Ambisome triggered different hemodynamic responses is not yet understood.

Summary:

a) Acute systemic hypotension developed after the first injections of Ambisome.

We compared the baseline and the minimum of SAP after 1st Ambisome injection using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%.

SAP decreased from a median of 146.5 mmHg to 81 mmHg ($p=0.0313$, $n=6$)

PAP change was not significant.

b) After the second identical dose of Ambisome (0.1 mg/kg) there were no hemodynamic changes.

We compared the baseline and the minimum of SAP after 2nd Ambisome injection using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%.

PAP and SAP did not change ($p=0.0625$ and $p=0.8438$, respectively, $n=6$).

c) The third, five-fold dose of Ambisome did not breach tolerance, and did not cause hemodynamic changes.

We compared the baseline and the minimum of SAP after 3rd Ambisome injection using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%.

PAP and SAP did not change ($p=0.0938$ and $p=1$, respectively, $n=6$).

d) Tachyphylaxis was confirmed by comparing baselines and maximally changed values of SAP (and PAP) between 1st and 2nd and 1st and 3rd injections.

We compared the baseline SAP before 1st and 2nd injection of Ambisome using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%. We also compared the maximally changed values after 1st and 2nd injection. We repeated the same test for comparison of the 1st and the 3rd injections, and also for the respective PAP values.

There was significant difference between baselines. Maximally changed SAP was lower after the 1st injection than after the 2nd ($p=0.0313$, 81 vs 147 mmHg, $n=6$) or 3rd injection ($p=0.0313$, 81 vs 147 mmHg, $n=6$). There were no other significant hemodynamic changes.

e) The positive control Zymosan provoked acute pulmonary hypertension in all cases, suggesting that tolerance induction by Ambisome was specific.

We compared the baseline and the maximum of PAP after Zymosan injection using Wilcoxon matched-pairs signed rank test, with a confidence interval of 95%.

The PAP increase was significant ($p=0.0313$, from 12 to 26 mmHg, $n=6$), while SAP remained unchanged.

Discussion

Complement activation related pseudo-allergy (CARPA) is a severe, life-threatening adverse event that may occur in some individuals upon administration of various drugs, nano-engineered formulations, radio contrast media, etc. Our overall goal was to develop an animal model that can help elucidate the mechanism and pathophysiology of the reactions, and to utilize this model to find a method to predict and ultimately to prevent these adverse events.

The pathomechanism of CARPA involves a cascade of cellular and molecular interactions. Complement activation leads to the generation of C3a, C5a and C5b-9, which trigger mast cells, basophils and other phagocytic cells via their specific receptors, and induce the secretion of a variety of vasoactive mediators. Some of these are preformed and released from the cells immediately upon activation, while others are de novo synthesized and released gradually. The effects of these allergomedins may explain the various symptoms of these reactions, e.g. vasoconstriction and vascular leakage mediated by H1 receptors may lead to the cardiovascular and cutaneous manifestations; H2 receptors in turn increase cellular cAMP levels and cause vasodilation increased heart rate and pulse pressure. An important factor in both the individual and the species dependent variation of these hypersensitivity reactions is the relative abundance of reactive cells in different organs of response, i.e. various parts of the vasculature, skin, lung, heart, bowel, etc.

Focusing on some of the cardiovascular symptoms, the rise or fall of SAP, for example, depends on the net impact of changes in cardiac output, pulmonary circulation and peripheral vascular permeability and resistance, just to mention the main factors. The decrease of cardiac output also has several contributing factors, such as impaired coronary circulation as a consequence of myocardial ischemia, bradycardia, reduced left atrial (and left ventricular) filling due to pulmonary vasoconstriction, and reduced afterload due to decrease of systemic vascular resistance. Pulmonary vasoconstriction, in turn, can arise as a consequence of arterial vasoconstriction due to thromboxane

liberation and pulmonary microembolisms, i.e. capillary blockade caused by aggregated and/or sequestered platelets and leukocytes. In an earlier study we provided pharmacological evidence for the liberation of adenosine in the coronary circulation, which likely contributes to bradycardia and systemic hypotension as a further aggravating factor. The formation and release of adenosine may explain a unique symptom of CARPA, paradoxical bradycardia, i.e. bradycardia despite severe arterial hypotension.⁵²

Another feature of CARPA is the decrease of exhaled CO₂, which is a distinctive factor compared to the known elevation of this parameter in asthma. The finding is rationalized with reduced elimination of CO₂ from blood at the alveolocapillary membrane due to decreased cardiac output, combined with pulmonary vasoconstriction and capillary blockage by microemboli. In asthma, on the other hand, CO₂ exchange at the alveolar level is unimpaired but CO₂ builds up in the exhaled air as a consequence of bronchoconstriction that restricts exhalation.

During our experiments we have tested the reactogenicity of the drug Doxil in pigs, and have found that the porcine model is extremely sensitive in terms of immunoreactivity to this liposomal formulation. The hemodynamic changes observed in the animals also correspond closely with the signs and symptoms presented by sensitive humans during clinical adverse events after administration of Doxil, and other potentially CARPA-genic agents. These include cough, dyspnea, chest pain, pulmonary edema, hypotension, fainting, light-headedness, dizziness, palpitations, angioedema, skin redness, rash, hives, itchiness, which can be explained with the acute pulmonary hypertension, increase or drop in systemic blood pressure, arrhythmias and/or ischemia, hypoventilation or apnea, and skin mottling observed in the porcine model.

However, it should be noted, that while all pigs exhibit CARPA to reactogenic liposomes (with 1 exception out of more than 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 hypersensitivity reactions to triggering liposomal drugs. Thus, pigs present a good model of only those (5–7%) humans who do react to Doxil.⁵³ Nonetheless the dose of Doxil that causes reactions in pigs corresponds to the human dose that reaches the circulation within a few seconds in reactive patients.

Therefore, the porcine CARPA test represents an appropriate model of these sensitive humans.

The *in vivo* model also features numerous unique benefits. The possibility to administer repeated doses of the test substances led to the discovery of tachyphylaxis, i.e. self-induced tolerance that is present upon repeated injections of Doxil.⁵⁴ We observed that after the second identical dose, and the third five-fold dose the reaction to the drug was substantially weaker or even not present at all.

The tachyphylaxis to Doxil raises the possibility of taking advantage of the phenomenon for the prevention of adverse, often severe hypersensitivity reactions to Doxil that may prohibit the use of the drug in certain susceptible individuals. Infusion reactions have also been observed during the phase I and II studies of Doxil.^{14,16} Several non-specific methods exist for preventing or reducing pseudoallergic reactions, including premedication with antihistamines and corticosteroids, but in severe cases discontinuation of drug administration may be necessary, sometimes with supportive therapy using bronchodilators or epinephrine. While partially effective, these none of these measures provide full protection against the hypersensitivity reactions. In the case of these tolerization promises major improvements in the application of these advanced drugs in the clinical setting.

Although this phenomenon is yet to be explained, it was already utilized for the development of a protocol that can prevent severe CARPA reactions in the porcine model.⁵⁵ We tested the hypothesis that slow, non-reactogenic infusion of Doxil-like but doxorubin-free liposomes, called “Doxebo”, may prevent the reaction to bolus doses of original Doxil. If the method will be perfected and proven to be applicable in hypersensitive human individuals in the future, they might also be able to benefit from drugs that are identified to have CARPA-genic potentials, like Doxil and many others.

Tachyphylaxis, i.e. a self-induced decrease of (patho)physiological response to a drug or agent has been observed with other substances, including opioids, LSD, nicotine, nitroglycerine, dobutamine, etc. However in those cases the decrease in response takes place over days or weeks. To our knowledge, the immediate and full tachyphylaxis described here for Doxil and Doxebio is not a commonly recognized

phenomenon in immunology, physiology, or pharmacotherapy. The short time course of the development of tolerance suggests that it is not related to active buildup of immune memory via specific cell activation. Consumption or depletion of complement factors can also be ruled out as the reactivity to strong C activators as our positive control Zymosan is maintained in the liposome-tolerized animals.

One hypothesis is that the mechanism of tachyphylaxis to PEGylated liposomes may involve the depletion of early mediators of CARPA, such as preexisting (natural) anti-PEG antibodies. The existence of these antibodies, particularly IgM, has been known and studied for some time.^{56,57} If they occur in low concentrations, it is reasonable to believe that they could be depleted upon the injection of the first dose of sufficiently large number of liposomes.⁵⁸⁻⁶²

Regarding tachyphylaxis, and the role of PEG, it should be noted that large PEI polymers did not induce similar complete tachyphylaxis in pigs as Doxil, which effect was slightly modulated by PEGylation of PEI.⁶³ Nonetheless, the small number of in vivo experiments limits our ability to draw solid conclusions.

Furthermore, the in vivo reaction to these polymers did not correlate with their C activation in human serum. The test we used for the measurement of in vitro complement activation by PEI polymers was based on an ELISA method for the detection of serum S-protein-bound C terminal complex (SC5b-9). LPS contamination as cause of in vitro C activation was ruled out and the validity of the test was corroborated by positive control samples. In 50% of the sera 80 mg/ml unmodified PEI 25 kDa caused significant elevation of SC5b-9 over PBS baseline, while the other polymers did not exhibit activity. Based on these measurements, the individual variation of SC5b-9 increase ranged approximately between 2 and 10-fold, and the effect of PEI 25 kDa was dose-dependent in the range between 20 and 80 mg/ml. In summary, we have found that only PEI 25 kDa showed major C activation in vitro, and in 2 out of 6 sera, none of the polymers caused significant C activation although the positive Zymosan control was active.

Nevertheless, all tested polymers showed cardiopulmonary reactogenicity in the porcine model, highlighting the variability in the sensitivity of various human sera and

calling for caution with the interpretation of the *in vitro* test results. This substantial individual variation is consistent with previous data on liposomal complement activation in multiple normal human sera.⁵³ PEI 25kD showed the highest reactogenic potential in the porcine model as well, and during the limited number of experiments with the polymers we have not observed complete tachyphylaxis. This calls into question the possibility of tolerization, and underlines the importance of the development of polymer formulations that have lower, or optimally no C-activating potentials. The fact that PEGylated polymers with no apparent *in vitro* C activation still caused reactions in pigs, suggest that the porcine CARPA test endpoints provide a more sensitive biomarker for anaphylactic reactions than the *in vitro* C tests. With our animal model we were able to identify the variants that were less reactogenic, providing hints for developers regarding what chemical properties and type of modifications might help to decrease reactogenicity. Based on the tendency observed, PEGylation with long PEG chains of >20 kDa may be favorable in the development of polyethylenimine-graft-poly(ethylene glycol) block copolymers for drug or nucleic acid delivery.

In light of the findings it is obvious that the porcine CARPA model is more sensitive than the *in vitro* ELISA assay. A possible explanation is that immune responses are multiplied by various cascades in the living individual but only to a certain extent in the serum *ex vivo*. The underlying cause of the high sensitivity of the pig model has not been clarified to date. The predominance of pulmonary symptoms suggests that the reactions may be related to the high number of intravascular macrophages (PIM cells) in the microcirculation of porcine lungs. As it has been outlined previously, the major hemodynamic changes entailing complement activation are most likely due to the release of thromboxane, other eicosanoids and leukotrienes, histamine and a range of further potent vasoactive substances from mast cells and basophil leukocytes upon binding of C3a and C5a to their respective receptors on these cells.

We have also preformed experiments in a dog model that is identical to the porcine model except for the species. The liposomal drug Ambisome provoked hemodynamic reactions in the subjects, and even a tachyphylacticphenomenon could be observed similarly to that during the porcine Doxil tests. However, the hemodynamic

changes had different characteristics compared to those following the injection of Zymosan, a known complement activator substance that is used as positive control in most C activation tests. This anomaly has yet to be explained and for this reason and because of the more extensive historic experience, we consider the porcine model to be superior and highly recommended at the moment.⁶⁴ This is also supported by its endorsement by the FDA.

The new generation of micro- and nano-sized drug delivery systems may bring an increased risk of recognition by the immune system as foreign.⁶⁵ It can be predicted that CARPA will be a returning safety problem in the upcoming age of nanomedicines. Hence, its understanding and prevention may become a critical step in the R&D of these agents.

Conclusions

1. First injection of Doxil causes clinically significant acute pulmonary arterial hypertension. The porcine model is extremely sensitive in terms of immune-reactivity to this liposomal formulation. The hemodynamic changes observed in the animals also correspond closely with the signs and symptoms presented by sensitive humans during clinical adverse events after administration of Doxil
2. There is tachyphylaxis for subsequent Doxil injections and they do not cause clinically significant acute pulmonary arterial hypertension or changes in systemic blood pressure. The tolerance is maintained even at a 5-fold dose.
3. Tolerization with Doxebo can be used to prevent severe CARPA reactions to Doxil. This raises the possibility of taking advantage of the phenomenon in the clinical setting. Prevention of the adverse, often severe hypersensitivity reactions may enable its application even in those susceptible individuals who otherwise would not be able to benefit from the drug.
4. Tolerance is specific to Doxebo and Doxil. The rapid development of tachyphylaxis suggests that it is not related to active buildup of immune memory via specific cell activation. Consumption or depletion of complement factors can also be ruled out as the reactivity to other strong C activators as Zymosan is maintained in the liposome-tolerized animals.
5. PEI-25kDa causes dose-dependent complement activation in vitro, while all PEI formulations induce CARPA in the pig model. PEI 25kD showed the highest reactogenic potential in the porcine model as well, and during the limited number of experiments with the polymers we have not observed complete tachyphylaxis
6. Dogs exhibit signs of CARPA when administered Ambisome, but these differ from the hemodynamic reactions in pigs after Doxil injections. The typical hemodynamic reaction in dogs to Ambisome is a drop in systemic arterial pressure without pulmonary arterial pressure changes, while to Zymosan the response is acute pulmonary hypertension without systemic arterial pressure change.

Summary

Several drugs and agents may cause hypersensitivity reactions (HSRs) that do not involve IgE but arise as a consequence of activation of the complement (C) system. These anaphylactoid reactions can be distinguished within the Type I category of HSRs as “C activation-related pseudoallergy” (CARPA). Drugs and agents causing CARPA include radiocontrast media, liposomal drugs (Doxil, Ambisome), micellar solvents (Cremophor EL), certain polymeric formulations (poly-ethylene-imine), carbon nanotubes and other nanoparticles. We have developed a porcine model for the investigation of the phenomenon, as minute amounts of reactogenic liposomes, such as Doxil, cause C activation in pigs with severe cardiovascular abnormalities that mimic some of the human symptoms.⁶⁴ During these tests we have seen an unusual form of tachyphylaxis that developed within minutes and was specific to Doxil-like liposomes.⁵⁴ This led to the formulation of Doxil-like empty liposomes, called Doxebo (placebo Doxil) and the development of a desensitization procedure that involves slow, low-dose infusion of Doxebo before Doxil treatment, which minimizes the ensuing physiological changes or keeps them subclinical.⁵⁵ Although the mechanism of tolerance induction is not yet clear, the effector arm of C response is unlikely to be affected, as the reactivity of desensitized pigs to the C activator zymosan remains intact. Desensitization with empty vesicles is a novel approach for reducing the risk of anaphylactic reactions to drug carrier liposomes, and potentially other CARPA triggering drugs. However, polyethyleneimine (PEI) polymers and PEGylated derivatives, although activated the C system, did not induce complete tachyphylaxis after repeated injection. The *in vitro* assays showed dose dependent C activation only by the high molecular weight PEI-25kDa, and none of the other types.⁶³ This suggests higher sensitivity of the porcine model⁶⁴, which may be particularly useful for the clarification of the mechanism of CARPA and tachyphylaxis⁵⁴, the refinement of the desensitization method⁵⁵, and the design of safe drug delivery systems⁶⁵.

Összefoglalás

Számos gyógyszeranyag (pl. liposzómásgyógyszerek (Doxil, Ambisome), micellárisoldószer (Chremophor EL), egyes polimerkészítmények (polietilénimin), karbonnanocsók, ésegyébnanorészecek) okozhatolyantúlérzékenységi reakciókat melyeket nem IgE-függőek, hanem a komplementrendszer aktiválásához kapcsolódnak. Ezek az anafilaktoid reakciók az 1-es típusú túlérzékenységi alcsoporthoz képezik a komplement aktivációs pszeudóallergia (CARPA) elnevezéssel. A jelenség vizsgálatára kidolgoztunk egy malacállat modellt, mivel csak a mennyiségű reaktogén liposzóma (pl. Doxil) is komplement aktivációt okozhat az állatokban súlyos kardiovaszkuláris kóvetkezményekkel, melyek megfelelnek az emberitüneteknek. Ezen kísérlet során megfigyeltük a tahifilaxis szokatlan formáját, ami percek alatt létrejött, és specifikus volt Doxilra és Doxil-szerű liposzómákra. Ez képezte az alapját a deszenzitizációs eljárásnak, mely során alacsony dózisú Doxil (vagy Doxil-szerű liposzóma) infúziót adunk az állatoknak a Doxil kezeléssel, ami megelőzte a reakciókat, illetve azok klinikailag nem manifestálódtak. Bár a tolerancia kialakulásának mechanizmusa egyelőren ismeretlen, úgy tűnik, hogy a komplementválasz effektorszakasza nem érintett, hiszen a deszenzitizált malacok továbbra is reagáltak a komplement aktiváló Zymosanra. Az úres liposzómákkal történő deszenzitizálást egy új megközelítés a CARPA-t kiváltó liposzómásgyógyszer hordozók kockázatainak csökkentésének, és esetleg más készítményeknél is alkalmazható. Ugyanakkor az általunk tesztelt polietilénimin polimerek és PEG-ált származékaik, bár mind komplement aktiváló hatásúknak bizonyultak, nem váltottak tahifilaxist a beadásukkor. Az in vitro teszt során azonban csak a nagymolekuláris súlyú, PEI-25kDa okozott dózis-függő komplement aktivációt. A sertésmodell a jóvábonkulásnak hasznos lehet a komplement aktivációs pszeudóallergia és a tahifilaxis vizsgálata, a deszenzitizáció módszerének értékeléséhez, és biztonságos gyógyszer hordozók kifejlesztésének szempontjából.

References

1. Coombs R, Gell PG. Classification of allergic reactions responsible for clinical hypersensitivity and disease. *Clinical Aspects of Immunology*; 1975.
2. Coombs R. The classification of allergic reactions underlying disease. *Clinical Aspects of Immunology*; 1962.
3. Descotes J. *Immunotoxicology of Drugs and Chemicals: Principles and methods of immunotoxicology*. Elsevier Science; 2004.
4. Portier P, Richet C. De l'action anaphylactique de certains venins. *C R Soc Biol*. 1902; 54:170–172.
5. Ring J, Behrendt H, de Weck A. History and classification of anaphylaxis. *Chem Immunol Allergy*. 2010; 95:1–11.
6. Schlumberger HD. Pseudo-allergic reactions to drugs and chemicals. *Ann Allergy*. 1983; 51:317–324.
7. Carroll MV, Sim RB. Complement in health and disease. *Advanced Drug Delivery Reviews*. 2011; 63:965–975.
8. Szebeni J, Fontana JL, Wassef NM, Mongan PD, Morse DS, Dobbins DE, Stahl GL, Bünger R, Alving CR. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. *Circulation*. 1999; 99:2302–2309.
9. Allen TM. Liposomes. Opportunities in drug delivery. *Drugs*. 1997; 54 Suppl 4:8–14.
10. Sculier JP, Coune A, Brassinne C, Laduron C, Atassi G, Ruyschaert JM, Frühling J. Intravenous infusion of high doses of liposomes containing NSC 251635, a water-insoluble cytostatic agent. A pilot study with pharmacokinetic data. *J. Clin. Oncol*. 1986; 4:789–797.

11. Levine SJ, Walsh TJ, Martinez A, Eichacker PQ, Lopez-Berestein G, Natanson C. Cardiopulmonary toxicity after liposomal amphotericin B infusion. *Ann. Intern. Med.* 1991; 114:664–666.
12. Ringdén O, Andström E, Remberger M, Svahn BM, Tollemar J. Allergic reactions and other rare side-effects of liposomal amphotericin. *Lancet.* 1994; 344:1156–1157.
13. Laing RB, Milne LJ, Leen CL, Malcolm GP, Steers AJ. Anaphylactic reactions to liposomal amphotericin. *Lancet.* 1994; 344:682–682.
14. Uziely B, Jeffers S, Isacson R, Kutsch K, Wei-Tsao D, Yehoshua Z, Libson E, Muggia FM, Gabizon A. Liposomal doxorubicin: antitumor activity and unique toxicities during two complementary phase I studies. *J. Clin. Oncol.* 1995; 13:1777–1785.
15. Alberts DS, Garcia DJ. Safety aspects of pegylated liposomal doxorubicin in patients with cancer. *Drugs.* 1997; 54 Suppl 4:30–35.
16. Dezube B. Safety assessment: Doxil (doxorubicin HCl liposome injection) in refractory AIDS-related Kaposi's sarcoma. Doxil clinical series; 1996.
17. Szebeni J. The interaction of liposomes with the complement system. *Crit Rev Ther Drug Carrier Syst.* 1998; 15:57–88.
18. Marceau F, Lundberg C, Hugli TE. Effects of the anaphylatoxins on circulation. *Immunopharmacology.* 1987; 14:67–84.
19. Hammerschmidt DE, Weaver LJ, Hudson LD, Craddock PR, Jacob HS. Association of complement activation and elevated plasma-C5a with adult respiratory distress syndrome. Pathophysiological relevance and possible prognostic value. *Lancet.* 1980; 1:947–949.
20. Szebeni J. Complement activation-related pseudoallergy: A new class of drug-induced acute immune toxicity. *Toxicology.* 2005; 216:106–121.

21. Rybak-Smith MJ, Sim RB. Complement activation by carbon nanotubes. *Advanced Drug Delivery Reviews*. 2011; 63:1031–1041.
22. Szebeni J, Muggia F, Gabizon A, Barenholz Y. Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: Prediction and prevention. *Advanced Drug Delivery Reviews*. 2011; 63:1020–1030.
23. Moghimi SM, Christy Hunter A, Dadswell CM, Savay S, Alving CR, Szebeni J. Causative factors behind poloxamer 188 (Pluronic F68, Flocor)-induced complement activation in human sera. A protective role against poloxamer-mediated complement activation by elevated serum lipoprotein levels. *Biochim Biophys Acta*. 2004; 1689:103–113.
24. Weiszhar Z, Czucz J, Révész C, Rosivall L, Szebeni J, Rozsnyay Z. Complement activation by polyethoxylated pharmaceutical surfactants: Cremophor-EL, Tween-80 and Tween-20. *Eur J Pharm Sci*. 2012; 45:492–498.
25. Hastings KL. Implications of the new FDA/CDER immunotoxicology guidance for drugs. *Int Immunopharmacol*. 2002; 2:1613–1618.
26. Why small matters. *Nat Biotechnol*. 2003; 21:1113.
27. Moghimi SM, Andersen AJ, Hashemi SH, Lettiero B, Ahmadvand D, Hunter AC, Andresen TL, Hamad I, jJ S. Complement activation cascade triggered by PEG-PL engineered nanomedicines and carbon nanotubes: the challenges ahead. *J Control Release*. 2010; 146:175–181.
28. Andersen AJ, Hashemi SH, Andresen TL, Hunter AC, Moghimi SM. Complement: alive and kicking nanomedicines. *J Biomed Nanotechnol*. 2009; 5:364–372.
29. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJA. Nanotoxicology. *Occup Environ Med*. 2004; 61:727–728.
30. Balbus JM, Maynard AD, Colvin VL, Castranova V, Daston GP, Denison RA,

- Dreher KL, Goering PL, Goldberg AM, Kulinowski KM, Monteiro-Riviere NA, Oberdörster G, Omenn GS, Pinkerton KE, Ramos KS, Rest KM, Sass JB, Silbergeld EK, Wong BA. Meeting Report: Hazard Assessment for Nanoparticles—Report from an Interdisciplinary Workshop. *Environ. Health Perspect.* 2007; 115:1654–1659.
31. Haxby JA, Kinsky CB, Kinsky SC. Immune response of a liposomal model membrane. *Proc Natl Acad Sci USA.* 1968; 61:300–307.
 32. Alving CR. Liposomal vaccines: clinical status and immunological presentation for humoral and cellular immunity. *Ann. N. Y. Acad. Sci.* 1995; 754:143–152.
 33. Duncan R. The dawning era of polymer therapeutics. *Nature Reviews Drug Discovery.* 2003; 2:347–360.
 34. Neuse EW. Synthetic Polymers as Drug-Delivery Vehicles in Medicine. *Metal-Based Drugs.* 2008; 2008:1–19.
 35. Mollnes TE, Brekke O-L, Fung M, Fure H, Christiansen D, Bergseth G, Videm V, Lappegård KT, Köhl J, Lambris JD. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood.* 2002; 100:1869–1877.
 36. Szebeni J, Baranyi L, Savay S, Götze O, Alving CR, Bünger R, Mongan PD. Complement activation during hemorrhagic shock and resuscitation in swine. *Shock.* 2003; 20:347–355.
 37. Linton S. Animal models of inherited complement deficiency. *Mol. Biotechnol.* 2001; 18:135–148.
 38. Redl H, Bahrami S. Large animal models: baboons for trauma, shock, and sepsis studies. *Shock.* 2005; 24 Suppl 1:88–93.
 39. Knight A. The beginning of the end for chimpanzee experiments? *Philos Ethics Humanit Med.* 2008; 3:16.

40. Bailey J. An examination of chimpanzee use in human cancer research. *Altern Lab Anim.* 2009; 37:399–416.
41. Lunney JK. Advances in swine biomedical model genomics. *Int. J. Biol. Sci.* 2007; 3:179–184.
42. Swindle MM, Smith AC, Hepburn BJ. Swine as models in experimental surgery. *J Invest Surg.* 1988; 1:65–79.
43. Shmeeda H, Even-Chen S, Honen R, Cohen R, Weintraub C, Barenholz Y. Enzymatic assays for quality control and pharmacokinetics of liposome formulations: comparison with nonenzymatic conventional methodologies. *Meth Enzymol.* 2003; 367:272–292.
44. Zuidam NJ, Barenholz Y. Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin. *Biochim Biophys Acta.* 1997; 1329:211–222.
45. Sakai H, Hisamoto S, Fukutomi I, Sou K, Takeoka S, Tsuchida E. Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus amoebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. *J Pharm Sci.* 2004; 93:310–321.
46. Binks BP. Modern characterization methods of surfactant systems. CRC Press; 1999.
47. Bellare JR, Davis HT, Scriven LE, Talmon Y. Controlled environment vitrification system: an improved sample preparation technique. *J Electron Microsc Tech.* 1988; 10:87–111.
48. Petersen H, Fechner PM, Fischer D, Kissel T. Synthesis, Characterization, and Biocompatibility of Polyethylenimine- graft-poly(ethylene glycol) Block Copolymers. *Macromolecules.* 2002; 35:6867–6874.
49. Szebeni J, Muggia FM, Alving CR. Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J Natl*

- Cancer Inst.* 1998; 90:300–306.
50. Szebeni J, Baranyi L, Savay S, Milosevits J, Bodó M, Bungler R, Alving CR. The interaction of liposomes with the complement system: in vitro and in vivo assays. *Meth Enzymol.* 2003; 373:136–154.
 51. Szebeni J, Baranyi L, Savay S, Bodo M, Morse DS, Basta M, Stahl GL, Bünger R, Alving CR. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. *Am J Physiol Heart Circ Physiol.* 2000; 279:H1319–28.
 52. Szebeni J, Baranyi L, Savay S, Bodó M, Milosevits J, Alving CR, Bungler R. Complement activation-related cardiac anaphylaxis in pigs: role of C5a anaphylatoxin and adenosine in liposome-induced abnormalities in ECG and heart function. *Am J Physiol Heart Circ Physiol.* 2006; 290:H1050–8.
 53. Chanan-Khan A, jJ S, Savay S, Liebes L, Rafique NM, Alving CR, Muggia FM. Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions. *Ann Oncol.* 2003; 14:1430–1437.
 54. Szebeni J, Bedocs P, Csukás D, Rosivall L, Bungler R, Urbanics R. A porcine model of complement-mediated infusion reactions to drug carrier nanosystems and other medicines. *Advanced Drug Delivery Reviews.* 2012; 64:1706–1716.
 55. Szebeni J, Bedocs P, Urbanics R, Bungler R, Rosivall L, Toth M, Barenholz Y. Prevention of infusion reactions to PEGylated liposomal doxorubicin via tachyphylaxis induction by placebo vesicles: A porcine model. *Journal of Controlled Release.* 2012; 160:382–387.
 56. Richter AW, Åkerblom E. Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. *Int. Arch. Allergy Appl. Immunol.* 1984; 74:36–39.
 57. Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, Garratty

- G. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer*. 2007; 110:103–111.
58. Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, Kiwada H. Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. *Journal of Controlled Release*. 2006; 112:15–25.
59. Ishihara T, Takeda M, Sakamoto H, Kimoto A, Kobayashi C, Takasaki N, Yuki K, Tanaka K-I, Takenaga M, Igarashi R, Maeda T, Yamakawa N, Okamoto Y, Otsuka M, Ishida T, Kiwada H, Mizushima Y, Mizushima T. Accelerated blood clearance phenomenon upon repeated injection of PEG-modified PLA-nanoparticles. *Pharm Res*. 2009; 26:2270–2279.
60. Koide H, Asai T, Hatanaka K, Akai S, Ishii T, Kenjo E, Ishida T, Kiwada H, Tsukada H, Oku N. T cell-independent B cell response is responsible for ABC phenomenon induced by repeated injection of PEGylated liposomes. *Int J Pharm*. 2010; 392:218–223.
61. Ishida T, Masuda K, Ichikawa T, Ichihara M, Irimura K, Kiwada H. Accelerated clearance of a second injection of PEGylated liposomes in mice. *Int J Pharm*. 2003; 255:167–174.
62. Ishida T, Maeda R, Ichihara M, Irimura K, Kiwada H. Accelerated clearance of PEGylated liposomes in rats after repeated injections. *Journal of Controlled Release*. 2003; 88:35–42.
63. Merkel OM, Urbanics R, Bedocs P, Rozsnyay Z, Rosivall L, Toth M, Kissel T, Szebeni J. In vitro and in vivo complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly(ethylene glycol) block copolymers. *Biomaterials*. 2011; 32:4936–4942.
64. Szebeni J, Alving CR, Rosivall L, Bungler R, Baranyi L, Bedocs P, Toth M, Barenholz Y. Animal models of complement-mediated hypersensitivity reactions to liposomes and other lipid-based nanoparticles. *Journal of liposome research*.

2007; 17:107–117.

65. Szebeni J, Bedocs P, Rozsnyay Z, Weiszár Z, Urbanics R, Rosivall L, Cohen R, Garbuzenko O, Bathori G, Toth M, Bungler R, Barenholz Y. Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome. *Nanomedicine : nanotechnology, biology, and medicine*. 2012; 8:176–184.

Author's Publications

On the subject of the dissertation:

1. Szebeni J, Bedocs P, Csukás D, Rosivall L, Bungler R, Urbanics R. A porcine model of complement-mediated infusion reactions to drug carrier nanosystems and other medicines. *Advanced Drug Delivery Reviews*. 2012; 64:1706–1716.
2. Szebeni J, Bedocs P, Urbanics R, Bungler R, Rosivall L, Toth M, Barenholz Y. Prevention of infusion reactions to PEGylated liposomal doxorubicin via tachyphylaxis induction by placebo vesicles: A porcine model. *Journal of Controlled Release*. 2012; 160:382–387.
3. Szebeni J, Bedocs P, Rozsnyay Z, Weiszhar Z, Urbanics R, Rosivall L, Cohen R, Garbuzenko O, Bathori G, Toth M, Bungler R, Barenholz Y. Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome. *Nanomedicine : nanotechnology, biology, and medicine*. 2012; 8:176–184.
4. Merkel OM, Urbanics R, Bedocs P, Rozsnyay Z, Rosivall L, Toth M, Kissel T, Szebeni J. In vitro and in vivo complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly(ethylene glycol) block copolymers. *Biomaterials*. 2011; 32:4936–4942.
5. Szebeni J, Alving CR, Rosivall L, Bungler R, Baranyi L, Bedocs P, Toth M, Barenholz Y. Animal models of complement-mediated hypersensitivity reactions to liposomes and other lipid-based nanoparticles. *Journal of liposome research*. 2007; 17:107–117.

On other subjects:

6. Buckenmaier CC III, Capacchione J, Mielke AR, Bina S, Shields C, Kwon KH, McKnight G, Fish DA, Bedocs P. The Effect of Lipid Emulsion Infusion on

Postmortem Ropivacaine Concentrations in Swine. *Anesth Analg.* 2012; 114:894–900.

7. Hood MN, Song T, Bedocs P, Capacchione JF, Kasper CE, Haigney MC, Ho VB. Multiacquisition T1-Mapping MRI During Tidal Respiration for Quantification of Myocardial T1 in Swine With Heart Failure. *American Journal of Roentgenology.* 2013; 201:W563–W570.

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