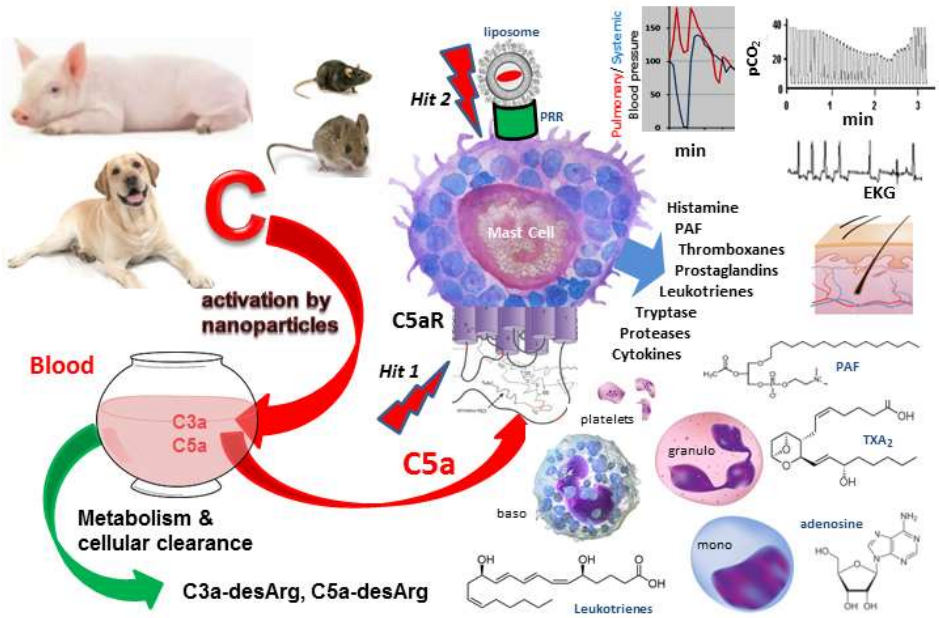
Hypersensitivity to polymer-coated nanomedicines: *Mechanism, prediction and prevention*

Gyula Tamás Fülöp



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HYPERSENSITIVITY TO POLYMER-COATED NANOMEDICINES: *MECHANISM, PREDICTION AND PREVENTION*

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General Introduction

Nanomedicine, a new era in healthcare

Nanomedicine, the medical application of nanotechnology, makes use of complex drugs and drug carrier molecular systems for advancing pharmacotherapy and diagnosis of human diseases. The field has been flourishing over the past decades and is expected to be a main contributor to human healthcare in the not-too-far future. As with all new frontiers in science, an initial hype is followed by a period of consolidation, the time of translation of utilizable scientific findings to clinical applications, which period might have arrived. This translation process involves a focus on the safety of nanomedicines, for which toxicity tests are needed. However, nanomedicines occasionally have unique, specific toxicities that have not been encountered in the past with traditional low-molecular weight drugs, requiring the development of new toxicity assays. This thesis focuses on an example for the above unique toxicity: hypersensitivity reactions (HSRs) to nanomedicines and their assessment in different assays.

Specific theme and goals

The dissertation focuses on those nanomedicines that are coated with polymers, and those HSRs that are triggered by a non-IgE-mediated process; complement (C) activation. Accordingly, it incorporates chapters with experimental studies and reviews on C activation-related pseudoallergy (CARPA) to nanomedicines.

The goals of the experimental studies (Chapters 2, 3) were to better understand the mechanism of specific reactions to some selected polymer-coated nanomedicines (PEGylated liposomes and dextran-coated SPIONs) and to explore possible methods for their prediction (by measuring C activation) and prevention (by slowing the infusion and using factor H). The reviews (Chapter 4, 6) on the other hand, give more comprehensive overviews on HSRs along with updates on specific subjects within this theme. The chapters of diverse topics are linked by their common background in nanotechnology, common classification of study systems as nanomedicines, and common focus of an adverse immune phenomenon: HSRs. These terms and concepts are defined and summarized as follows.

Description of terms and concepts

Nanotechnology is the manipulation of complex atomic, molecular, and macromolecular structures and systems within the nanometer scale (10^{-6}-10^{-9} m) to achieve a novel or superior characteristic or property.[1] ***Nanodrugs***, also known as ***Nanomedicines*** are nanoparticular drugs or diagnostic agents with broad and expanding applications including targeted drug delivery and in vivo imaging. A class of nanomedicines, to which natural or synthetic polymers are attached via covalent bondage, is referred to as ***polymer coated nanomedicines*** wherein the polymers endow advantageous properties to the nanodrug, such as stability in aqueous solutions, extended circulation time and capability for passive or active targeting of active pharmaceutical ingredients (API) to cellular sites of disease. The chemical composition and steric structure of nanomedicines substantially differ in subcategories, such as micelles, liposomes, solid lipid nanoparticles, polymers, polymer conjugated proteins/antibodies, nano-crystals, dendrimers, carbon nanotubes, buckyballs, etc. ***Liposomes and solid lipid nanoparticles*** are vesicular and solid (phospho)lipid assemblies, respectively, with capability to entrap and carry drugs. Their buildup from non-toxic lipids, i.e., cholesterol and natural or synthetic phospholipids and adaptivity with regard to size, surface charge and conjugated surface ligands make them useful products in the market. [2-4] ***SPIONs*** are superparamagnetic iron oxide nanoparticles in the 10-100 nm range which contain a γ-Fe_2O_3 (maghemite), Fe_3O_4 (magnetite) or α-Fe_2O_3 (hermatite) core and a hydrophilic surface coating made from a variety of polymers, including dextran, carboxydextran, chitosan, phospholipids, PEG and starch. SPIONs are best known as contrast agents in MRI, with several products reaching the market. However, they also have potential applications in cancer and antibacterial chemotherapy, oral delivery of nano-vaccines and central nervous system injury repair. [5,6] ***Monoclonal antibodies*** (mAbs), [7] along with other products of biological origin, are increasingly used in pharmacotherapy, although only their complexed or surface-conjugated versions are classified as nanomedicine, not their monomolecular formulation. The relevance of the latter in the present dissertation lies in the fact that they too cause HSRs with basic resemblance to those caused by nanomedicines. The modification of nanomedicines and biological molecules with a ***polymer coating*** refers most often to covalent conjugation with ***polyethylene glycol (PEG),***[8] i.e., linear polymers of ethylene oxide in the 0,8- 20 kDa MW range, lending solubility, stability and increased biocompatibility of the nanocarrier materials. [7-8]

Besides advantages at the level of efficacy and/or toxicity of associated drugs or diagnostic agents, these and many other nanomedicines can cause ***HSRs***, a sort of capricious adverse immune effect also known as ***infusion reaction, or anaphylactoid reaction***. The clinical manifestations

include individually variable combinations of more or less severe allergy symptoms which, in a few percentages of cases, can culminate in ***anaphylactoid (cardiac) shock***. In clinical medicine, these severe HSRs are referred to as ***severe adverse events***, a dreaded health hazard that may entail death and therefore suspension of the drug along with major unfavorable legal and socio-economic consequences. Although nanomedicine-induced HSRs are mostly transient and tolerable and their severity can be alleviated by anti-inflammatory premedication, severe HSRs still surface from time to time in clinical studies and post-marketing surveillance. Therefore, this problem remains to represent a significant barrier to the clinical application of many promising nanomedicines, giving strong endorsement for scientific studies to address this problem.

Complement activation related pseudo allergy (CARPA) is a mnemonic name for HSRs that are caused by activation of the C system, or have C activation involved in the beginning of their emergence. Some nanomedicines, such as liposomes, because of their remembrance to viruses, often have C activating properties. [9-12] In addition to HSRs, C activation may hurt the therapeutic potential of liposomes in 2 additional ways: 1) it can lead to opsonization of vesicles, which, in turn, triggers their rapid clearance, 2) it can augment the immunogenicity of liposomes, which makes their repeated use problematic. Clinically used liposomal drugs reported to cause HSRs include Doxil (Caelyx), Myocet, Abelcet, AmBisome, Amphotec, DaunoXome, Visudyne, Onivyde, Vyxeos, Onpattro). In addition to liposomes, CARPA can be caused by micellar drugs (e.g., Taxol, Taxotere, Etoposide), radiocontrast media (e.g., Diatrizoate, Iodixanol, Iohexol, Iopamidol, Iopromide, Iothalamate, Ioversol, Ioxaglate, Ioxilan, SonoVue, Magnevist), biologicals (e.g., Avastin, Enbrel, Herceptin, Humira, Raptiva, Synagis, Xolair, Compath, Erbitux, Mylotarg, Remicade, Rituxan,Vectibix, Tysabri), enzymes (Avonex, Actimmune, Abbokinase, Aldurazyme, Activase, Zevalin, Neupogen, Neulasta, Fasturec, Plenaxis), and miscellaneous other drugs, (e.g., Cancidas, Copaxone, Orencia, Eloxatin, Salicilates, analgetics, morphine). Unpredictable HSRs are becoming a major concern for drug companies, as regulatory agencies increasingly emphasize the need for new, non-standard toxicity tests that enable prediction of adverse immune consequences of nanomedicine administration. A recent example is the recommendation by the Committee for Human Medicinal Products of the European Medicines Agency (EMA) to “use in vitro and in vivo immune reactogenicity assays, such as complement (and/or macrophage/basophil activation assays) and testing for “CARPA” in the case of (generic) liposomal products. Furthermore, the FDA also recommends to study C activation in vitro and/or in vivo as an immunotoxicology test to identify drugs with risk to trigger pseudoallergy. [13]

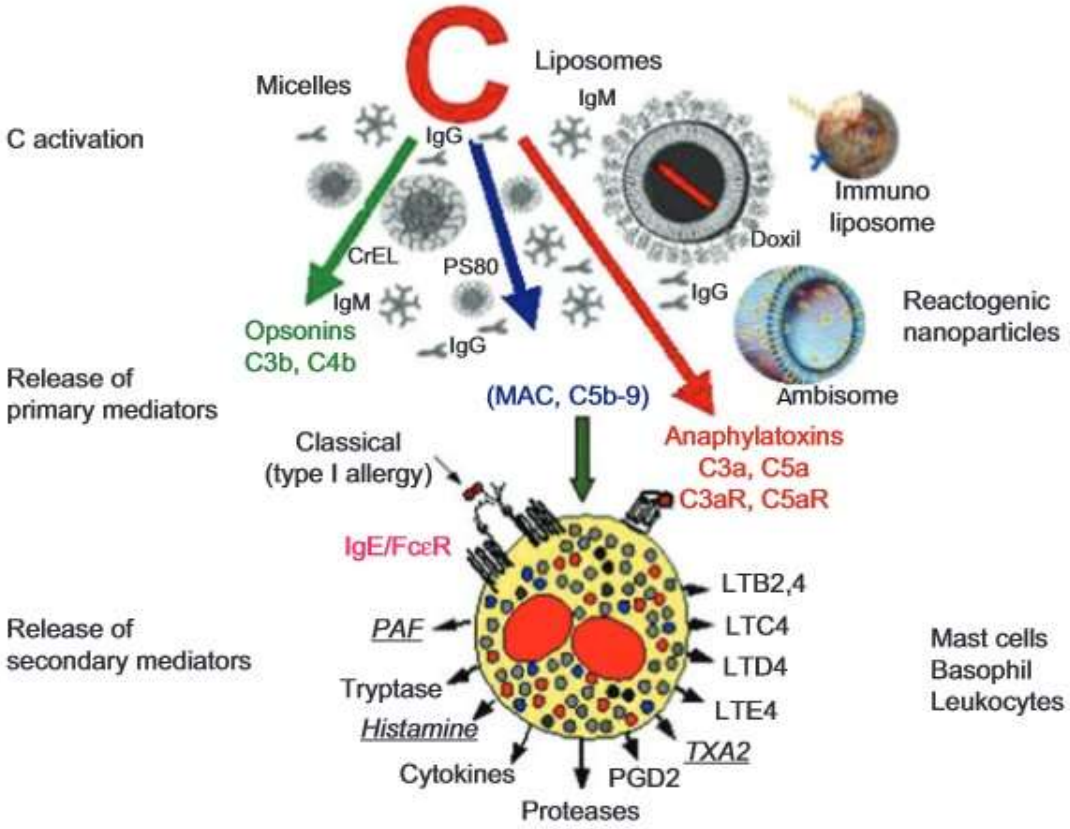

Figure 1. Mechanism of CARPA: several nanomedicines like liposomes, nanoparticles, nanotubes, even certain monoclonal antibodies are capable of causing HSRs triggered by complement activation. The quick acting reaction to certain nanomedicines induces the release of several types of primary mediators: anaphylatoxins which amplify and direct the complement and also other participants of the immune system such as mast cells, basophils and leukocytes to release secondary mediators like TxA2 (thromboxane), cytokines (interferons and interleukins), histamine etc. that produce allergy symptoms. [14]

Although CARPA resolves in most patients within minutes or hours after stopping the infusion, the reaction may become life-threatening in a minority of patients (a few percent) and, occasionally, become even fatal (roughly at ≤0.01%). Many of the symptoms of CARPA are common manifestations of classical IgE (Immunoglobulin E) mediated type-I allergy: angioedema, asthma attack, bronchospasm, chest pain, chill, choking, confusion, dyspnea, edema, erythema, headache, hypertension, hypotension, hypoxemia, low back pain etc. The features of CARPA that distinguish them from classical IgE-mediated reactions include 1) the rise of symptoms at first exposure; 2) the diminution or disappearance of symptoms upon re-exposure; 3) their spontaneous resolution; 4) the dependence of reaction strength on the speed of infusion; 5) their response to steroid and

antihistamine premedication; 6) the high reaction rate (2–10%) and, finally, 7) the negativity of standard allergy tests. [9] The exact mechanism of CARPA differs for each nanomedicine, meaning that the pathways leading to similar symptoms may differ from one nanostructure to another.

PEGylated liposomal prednisolone phosphate is a nanodrug that we used in some of our studies as a model for PEGylated liposomes. It was developed for the treatment of chronic inflammatory diseases, including inflammatory bowel disease, rheumatoid arthritis, ulcerative colitis and multiple sclerosis. It reached Phase 2A clinical trials in which it showed significant efficacy. As adverse event prevention is always preferred over trying to 'cure' the suddenly arisen unwanted reaction(s), different infusion protocols designed to differ in rate of infusion of the nanomedicine were studied and compared in a porcine model. Several biological parameters that indicate C activated HSR is studied during these in vivo experiments, following haemodynamic changes as heart rate, systemic arterial pressure, pulmonary arterial pressure, respiration (pCO2, SpO2) and from blood samples taken at several time-points blood cell analysis and several biomarker (TxB2, FH, sC5B9) can be measured with ELISA techniques.

SPIONS (dextran-coated superparamagnetic iron-oxides) are also examples of polymer-coated nanomedicines that can possibly trigger a C activation related HSR. These useful constructs used in diagnostics and also therapy were analyzed for their physicochemical features and reactogenicity to find possible correlations. With this knowledge, possibly less reactogenic SPIONs can be developed in the future. We also highlight the importance of using adequate methods for performing the physicochemical characterization of nanoparticles, particularly with regards to quantifying inhomogeneity and detecting aggregates to prevent HSRs.

Furthermore, studying possible biomarkers that are involved in C activation triggered by polymer-coated nanomedicines seems a logical first step to develop feasible biomarker assays for prediction and prevention of HSRs. Examples are:

Thromboxane A2 (TXA2) is an eicosanoid lipid derivative with numerous powerful biological effects, including pulmonary and coronary vasoconstriction, i.e., prominent manifestations of severe CARPA. Therefore, a review is written and included assembled about the role of thromboxane in CARPA reactions. To our experience to date, none of the blood tests currently available correlate better with the hemodynamic changes in CARPA than the TXB_2 assay, providing a valid endpoint for in vivo screening assays for regulatory evaluation of the CARPAgenic effect of drugs under R&D.

Complement factor H (FH) is a well-known natural inhibitor of C activation via the alternative pathway. FH was theorized to have a possible role in the occurrence of HSRs triggered by polymer-coated nanomedicines as well as polymer-coated (PEGylated) monoclonal antibodies. The formation of ***anti-PEG IgMs*** against PEG molecules on liposomes has been demonstrated in *in vivo* CARPA studies. In case of PEGylated monoclonal antibodies, similar immunological responses producing anti-PEG IgMs may occur.

A possible approach to prevent or reduce the chance of the occurrence of CARPA is the administration of complement inhibitors just prior to or at the same time of the administration of the therapeutic agent in question. Even though this could be a good option as a prevention modality, most patients do not need such an action as they are not prone to HSRs, and therefore this option would just elevate the therapy costs. The best scenario would be to pre-screen each patient for proneness to any adverse hypersensitivity reaction, using an *in vitro* test performed on a blood sample of the patient, to predict if any CARPA event could arise during or after administration of a therapeutic agent, such as a polymer coated nanomedicine and monoclonal antibody.

Aim and outline of the thesis

The aim of the research described in this thesis is to investigate the mechanism, prediction and prevention of CARPA-related hypersensitivity reactions to polymer-coated nanoparticles. To fulfill this purpose, we have focused on the following specific questions regarding the CARPA-related hypersensitivity to polymer-coated nanomedicines:

1. Can the risk for occurrence of CARPA-related HSRs to PEG-liposomal prednisolone be reduced by modifying the infusion protocol? (Chapter 2)
2. What is the role of complement (C) activation in the triggering of CARPA related HSRs to PEGylated low-molecular-weight dextran-coated superparamagnetic iron-oxides (SPIONs)? Does the reactogenicity of these nanoparticles correlate with their physicochemical features? (Chapter 3)
3. What is the role of TXA2 in CARPA? (Chapter 4)
4. What is the role of complement factor H in CARPA? (Chapter 5)
5. Can complement factor H protect against CARPA reactogenicity resulting from the administration of PEGylated monoclonal antibodies? (Chapter 6)

Finally, Chapter 7 provides a summary and discussion of the findings in this thesis, including perspectives on future implications.

References

1. Bawa, R., Bawa, S. R., Mehra, R. (2016). The translational challenge in medicine at the nanoscale. In: Bawa, R., ed.; Audette, G. F., Reese, B. E., asst. eds. Handbook of Clinical Nanomedicine: Law, Business, Regulation, Safety, and Risk, Pan Stanford Publishing, Singapore, chapter 58, pp. 1291–1346.
2. Akbarzadeh, A. et al. in Nanoscale Res Lett Vol. 8 102 (2013).
3. Sharma, A. & Sharma, U. S. Liposomes in drug delivery: Progress and limitations. International Journal of Pharmaceutics 154, 123-140, doi:https://doi.org/10.1016/S0378-5173(97)00135-X (1997).
4. Bozzuto, G. & Molinari, A. in Int J Nanomedicine Vol. 10 975-999 (2015).
5. Kakarla, R. Functionalized magnetic nanoparticles/biopolymer hybrids: Synthesis methods, properties and biomedical applications. Methods in microbiology vol. 46 (2019) https://doi.org/10.1016/bs.mim.2019.04.005
6. Mahmoudi, M. Sant, S. Superparamagnetic iron oxide nanoparticles (SPIONs): Development, surfacemodification and applications in chemotherapy. Advanced drug delivery reviews vol. 63 (2011)
7. Veronese, F. M. & Mero, A. The impact of PEGylation on biological therapies. BioDrugs 22, 315-329 (2008).
8. Immordino, M. L., Dosio, F. & Cattel, L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomedicine 1, 297-315 (2006).
9. Alving, C. R., Kinsky, S. C., Haxby, J. A. & Kinsky, C. B. Antibody binding and complement fixation by a liposomal model membrane. doi:10.1021/bi00832a038 (2002).
10. Kinsky, S. C., Haxby, J. A., Zopf, D. A., Alving, C. R. & Kinsky, C. B. Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. Biochemistry 8, 4149-4158 (1969).
11. Szebeni, J. & Storm, G. Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs. Biochemical and Biophysical Research Communications 468, 490-497, doi:10.1016/j.bbrc.2015.06.177 (2015).

12. Szebeni, J. Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. Toxicology 216, 106-121, doi:10.1016/j.tox.2005.07.023 (2005).
13. Hastings, K. L. Implications of the new FDA/CDER immunotoxicology guidance for drugs. International Immunopharmacology 2, 1613-1618, doi:https://doi.org/10.1016/S1567-5769(02)00061-9 (2002).
14. Szebeni, J. Hemocompatibility testing for nanomedicines and biologicals: predictive assays for complement mediated infusion reactions. doi:https://doi.org/10.1515/ejnm-2012-0002

2

Liposome-induced hypersensitivity reactions: risk reduction by design of safe infusion protocols in pigs

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Abbreviations:

CARPA, complement activation-related pseudoallergy
HR, heart rate
HSRs, hypersensitivity reactions
PL, phospholipid
PAP, pulmonary arterial pressure
SAP, systemic arterial pressure

Abstract

Intravenous administration of liposomal drugs can entail infusion reactions, also known as hypersensitivity reactions (HSRs), that can be severe and sometimes life-threatening in a small portion of patients. One empirical approach to prevent these reactions consists of lowering the infusion speed and extending the infusion time of the drug. However, different liposomal drugs

have different levels of reactogenicity, which means that the optimal protocol for each liposomal drug may differ and should be identified and evaluated to make the treatment as safe and convenient as possible.

The goal of the present study was to explore the use of pigs for that purpose, using PEGylated liposomal prednisolone (PLP) as a model drug. We compared the reactogenicities of bolus versus infusion protocols involving 2-, 3- and 4-step dose escalations for a clinically relevant total dose, also varying the duration of infusions. The strength of the reaction was measured via continuous recording of hemodynamic parameters and blood thromboxane B2 levels. We showed that bolus administration or rapid infusion of PLP caused transient changes in systemic and pulmonary blood pressure and heart rate, most notably pulmonary hypertension with paralleling rises in plasma thromboxane B2. These adverse responses could be significantly reduced or eliminated by slow infusion of PLP, with the 3-h 3-step dose escalation protocol being the least reactogenic. These data suggest that the pig model enables the development of safe infusion protocols for reactogenic nanomedicines.

Key words: Infusion reaction; complement; anaphylatoxins; pseudoallergy; CARPA; PEGylation; nanoparticle, nanopharmaceuticals; nanomedicines

Introduction

Non-IgE-mediated (pseudoallergic) hypersensitivity, or infusion reactions (IRs) following i.v. administration of nanomedicines and biologicals are infrequent but salient adverse immune effects of many state-of-art pharmaceuticals, including PEGylated liposomes, such as liposomal doxorubicin (Doxil). Earlier studies provided evidence that the cardiovascular and cutaneous symptoms of human IRs to liposomes, in general, and Doxil, in particular, can be reproduced in pigs by i.v. injection of these liposomal drugs in pigs. Moreover, it has been established that the symptoms can be explained by complement activation, hence the name "complement activation-related pseudoallergy" (CARPA) [1].

It was also shown earlier in pigs that the rate of infusion of multilamellar liposomes had a significant impact on their reactogenicity, with slowing the infusion speed leading to reduced cardiopulmonary distress [2]. As this is concordant with the human experience that slowing the infusion rate reduces the risk of HSRs [3], these observations suggest that pigs can be used to

model the impact of infusion speed on human HSRs and to develop safe infusion protocols. The goal of the present study was to explore this possibility, using PEGylated liposomal prednisolone sodium phosphate (PLP) as model for PEGylated liposomes and testing therapeutically relevant 2- and 3-h infusion protocols versus bolus i.v. administrations. Indeed, PLP was shown earlier to cause C activation in human serum [4], suggesting the potential for causing HSR in man. Thus, the present experiments also served the purpose of developing a safe administration protocol for PLP.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-poly-ethyleneglycol conjugate-2000 (DSPE-PEG2000) and cholesterol were obtained from Lipoid GmbH, Ludwigshaven, Germany). Prednisolone sodium phosphate was from BUFA (Uitgeest, The Netherlands) and Zymosan from Sigma. The TXB2 kit was from Amersham (UK).

Preparation of liposomes

PEGylated liposomal prednisolone sodium phosphate (PLP) was prepared using the ethanol injection method [5] encapsulating prednisolone sodium phosphate (Bufa, Uitgeest, the Netherlands) with DSPE-PEG2000, DPPC and cholesterol (Chol, Sigma, St Louis, USA) in a 0.15:1.85:1.00 molar ratio. Multiple rounds of extrusions through polycarbonate membranes (final pore sizes of 100nm, Nucleopore, Pleasanton, USA) were performed and unencapsulated prednisolone was removed with a tangential flow filtration unit (Pall Minimate, Pall Millipore). Mean particle size was determined using dynamic light scattering (DLS) and the amount of encapsulated prednisolone and free (unencapsulated) prednisolone was determined with high performance liquid chromatography as described previously [5,6]. The encapsulated prednisolone sodium phosphate content was 2.8 mg/mL and the unencapsulated prednisolone remained under 0.02 mg/mL. The mean size was 100 nm, polydispersity index ~0.1 and zetapotential ~ -5 mV in PBS.

CARPA studies in pigs

Details of the pig experiments were described earlier [1, 7, 8]. In brief, mixed breed male Yorkshire/Hungarian White Landrace pigs (2-3 months old, 20-25 kg) were obtained from the Animal Breeding and Nutrition Research Institute, Herceghalom, Hungary. Animals were sedated with Calypsol/Xilazine and then anesthetized with isoflurane (2–3% in O_2). Intubation was performed with endotracheal tubes to maintain free airways, and to enable controlled ventilation if necessary. The animals were breathing spontaneously during the experiments. Surgery was done after povidone iodine (10%) disinfection of the skin. In order to measure the pulmonary arterial blood pressure (PAP), a Swan–Ganz catheter (AI-07124, 5 Fr. 110 cm, Arrow Internat Inc.) was introduced into the pulmonary artery via the right external jugular vein. Additional catheters were placed into the left femoral artery to record the systemic arterial pressure (SAP), to the left external jugular vein for saline and drug administration, and to the left femoral vein for blood sampling. Before and during infusion pigs were monitored for PAP, SAP and heart rate (HR) changes, among many other parameters that are customarily measured in our model [1, 7, 8], but were not presented in this paper as their changes were consistent with those of PAP. The latter was expressed both in absolute and relative terms (compared to baseline), or as area under the curve during the first 15 min of the first reaction (AUC), which measure was independent of individual variation of PAP waveforms. Blood samples were collected pre-administration and at various times post-administration for the measurement of plasma thromboxane B2 (TxB2).

PLP administration via different infusion protocols

From the 18 pigs used in this study, 3 obtained PLP as a bolus IV administration and the rest of animals were treated with different PLP infusion protocols. The total drug dose was equal in all pigs, 3 mg/kg, which corresponded to the human therapeutic dose. In case of infusion appropriate volumes from the stock (provided in sterile vials) containing 2.3 mg PLP/mL were diluted in 5.5 volume normal saline (NS). Upon treatment, animals were randomly selected into 3 groups differing in the speed and length of infusion (see Table 1). At the start of each experiment, animals received 5 mL NS (baseline), injected as i.v. bolus, and then they were monitored for 5-10 minutes before starting the infusion.

Statistical methods

Normality was tested by the Kolmogorov-Smirnov test. The PAP and TxB2 values at all time points were compared to their baseline (0 min) and the significance of differences were determined by non-parametric Kruskal-Wallis and Friedman test, followed by Dunn's multiple comparisons. A p-value of <0.05 was considered to be statistically significant. Statistical analysis was performed by GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Results

Effects of PLP administered as a repeated bolus

Among the common symptoms of CARPA in pigs (changes in hemodynamic, respiratory, hematological, and blood chemistry parameters and skin alterations), we previously found the hemodynamic and TxB2 alterations to be the most reproducible and quantitative. Therefore, we focused on these changes in PLP-treated pigs.

Fig. 1 shows the changes in PAP, SAP and HR caused by repetitive bolus injections of PLP; real-time tracings in 3 different pigs (panels A-C) to illustrate the individual variation. The injected doses are specified above the arrows which show the time of injection. The second injection repeated the first, and then the subsequent (3^{rd}) dose was increased 5-fold in order to establish any change in sensitivity, and, hence, the validity of using the peak heights for quantitation of HSRs in case of repeated injections. In all 3 pigs the first bolus led to massive (300-600%) rises of PAP, which was followed by no, or smaller changes after an identical, and then larger repeat doses. Thus, the PAP response to PLP was tachyphylactic (self-limiting); therefore, only the first peak was taken as quantitative measure of the drug's reactivity. The SAP and HR showed less or no changes after each injection, while Zymosan (0.1 mg/kg), used as positive control, caused massive pulmonary hypertension in each animal. These changes were consistent with other liposome reactions described in this model [1, 7, 8], particularly those induced by PEGylated liposomal doxorubicin (Doxil) and its drug-free equivalent vesicle (Doxebo) [9, 10]. Our results therefore suggested that the reaction is due to the liposomal bilayer, and not to the drug payload. Likewise, the individual variation of first peaks is consistent with our previous results with Doxil [9, 10].

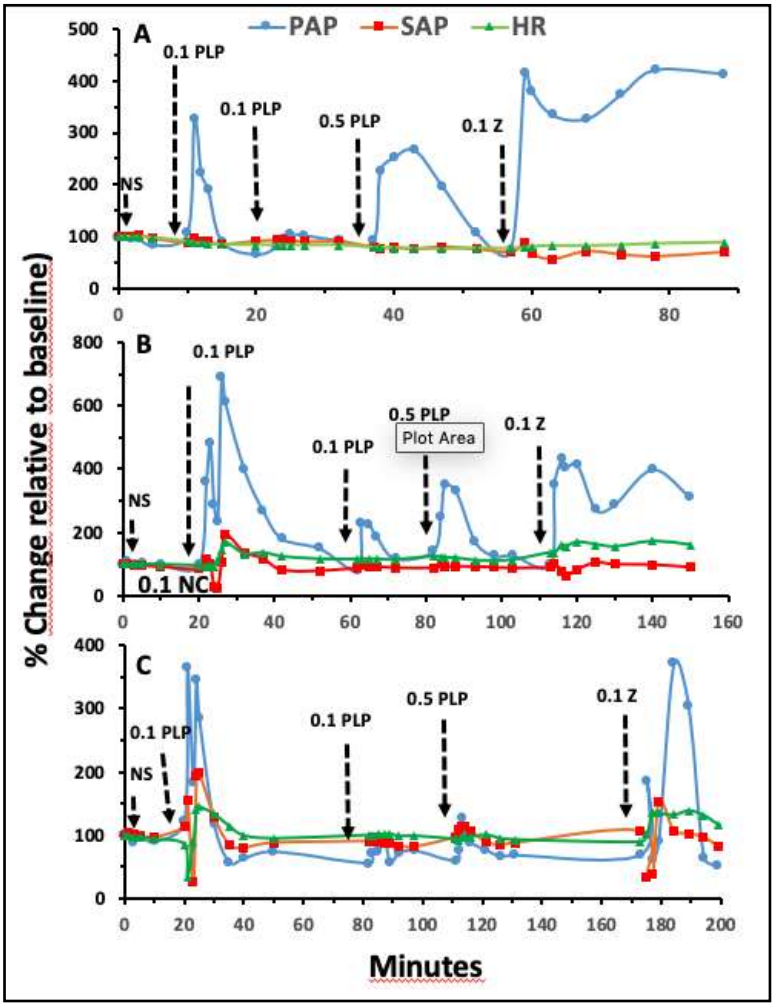

Figure 1. Real-time recordings of the hemodynamic effects of PLP boluses in 3 different pigs (A-C). Pulmonary arterial pressure (PAP), systemic arterial pressure (SAP) and heart rate (HR) changes are expressed as % of baseline. The time of i.v. injections are indicated by arrows. NS, normal saline. The numbers before PLP specify the phospholipid dose (mg/kg). 0.1 Z, 0.1 mg/kg zymosan. Other details are described in the Methods.

The above data suggest that the features of cardiopulmonary reaction caused by PLP are very similar to those described for liposomal doxorubicin (Doxil) [9, 10].

Effects of PLP administered in infusion: impacts of infusion rate, duration and drug dose

Next we examined the reactogenicity of PLP administered in infusion using different protocols referred to as A, B and C (Table 1 and 2). These protocols represented stepwise increases of dose rate over different time windows and different overall duration of infusion. In protocols A, B and C the dose rates were increased in 4, 2 and 3 steps, respectively, with major differences in total infusion time and dose rates at the first step.

Table 1. Parameters of PLP infusion in pigs in 3 administration protocols

Steps	**Step 1**			**Step 2**			**Step 3**			**Step 4**			**time**
Protocol (n pigs)	**min**	**rate ml/kg/h**	**total mL/kg**	**min**	**rate ml/kg/h**	**total mL/kg**	**min**	**rate ml/kg/h**	**total mL/kg**	**min**	**rate ml/kg/h**	**total mL/kg**	**min**
A (n=1)	**15**	**0.24**	**0.07**	**15**	***0.60***	**0.15**	**15**	**1.20**	**0.30**	**90**	***6.00***	**9.00**	**135**
B (n=10)	**20**	**0,40**	**0.13**	**115**	***4.00***	**7.67**		**0**	**0**				**135**
C (n=5)	**40**	**0.04**	**0.03**	**20**	**0.40**	**0.13**	**120**	***4.00***	**8.0**				**180**

Abbreviations: mL refers to undiluted PLP stock containing 2.3 mg/mL prednisolone sodium phosphate and 37.5 mg/mL (50 mM) phospholipid (see Methods). Infusion was done after 5.5-fold dilution of PLP stock in NS. For simplicity, the 135 min infusion times are referred to as 2-h protocol thenceforth. Bold italicized entries triggered more or less pulmonary hypertension, as specified below.

Table 2. Phospholipid dose rates in the different steps of different protocols

protocol	**Infusion steps**			
	1	2	3	4
	mg phospholipid/kg/min			
A	0.17	***0.38***	0.75	***3.75***
B	***0.24***	***2.50***		
C	0.03	0.24	***2.50***	

The entries were obtained from Table 1 by dividing the amount of phospholipid injected during the different steps by the duration of steps. Bold italicized entries triggered more or less pulmonary hypertension, as specified below.

As shown in Fig 2A, the rises of infusion rates to 0.38 and then to 3,75 mg PL/kg/min) at the 2nd and 4th step in protocol A triggered major, permanent (up to 300%) rise of PAP, indicating significant cardiopulmonary distress. Since our aim was to prevent these changes, this protocol was not tested in further animals. In protocols B and C, applied in 10 and 5 pigs, respectively (Figs 2B and C), the increases of infusion rates to 2.5 mL/kg/h at the 2nd or 3rd steps, respectively, also caused permanent pulmonary hypertension, but these were less prominent (20% and 50% rises, respectively, Figs 2B and C). The 0.24 mg phospholipid/kg/min initial infusion in protocol B (0.24 mL/kg/h) still caused a moderate but significant rise of PAP (Fig 2B, Table 3), while an 8-fold reduction of the initial dose rate in protocol C was reaction free at the first and second steps of dose escalation, with minor, biologically negligible rise of PAP after the 3rd infusion step (Fig. 2C, Table 3).

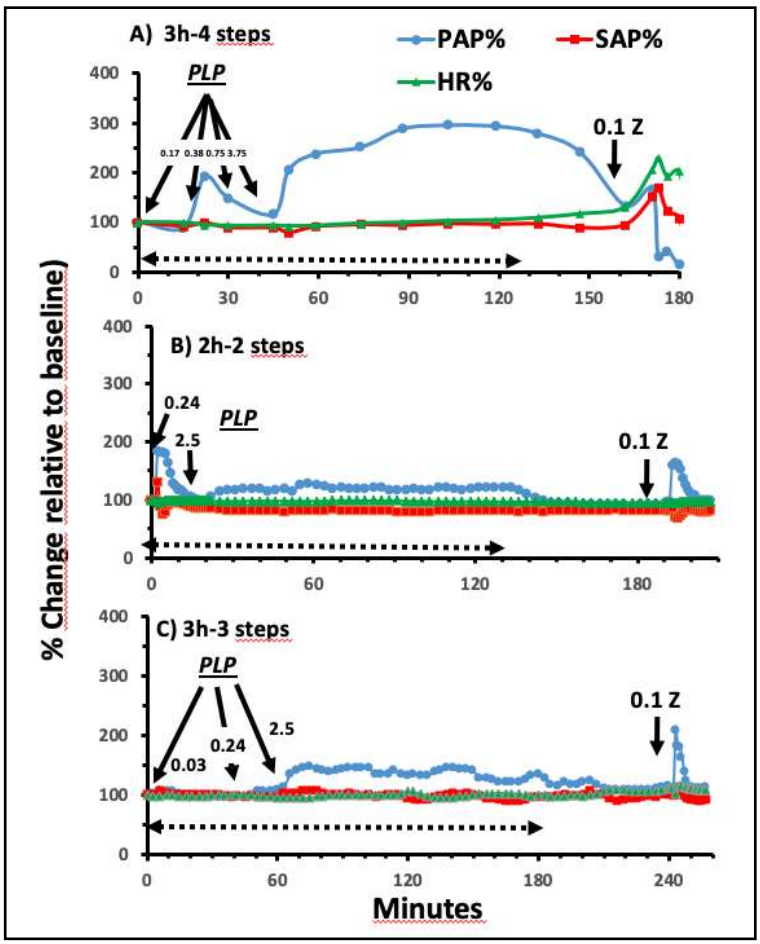

Figure 2. Real-time recordings of the hemodynamic effects of PLP infusion according to 3 administration protocols, specified in Table 1 and 2. All other details are the same as in Fig. 1, except that dotted lines are used to show the length of infusion and the rates of infusion are specified, instead of infusion dose (Fig. 1 was bolus treatment). Protocol A was applied only in 1 pig. Panels B and C show typical data from 9 and 4 animals infused with the 2- and 3-step protocols, respectively, with 1-1 outlier (identified by the Kolmogorov-Smirnov test) excluded from both groups.

Table 3. Initial (within 10 min) rise of PAP induced by the 2-step (B) and 3-step (C) dose escalation infusion protocols for PLP administration.

Protocol	pig n	mean % of baseline	SD	SEM
B (2-step)	9	140.0*	39.8	12.6
C (3-step)	4	105.7	2.8	1.4

*Significant difference relative to baseline and protocol C, Mann Whitney P: 0.007.

These data taken together suggest that stepwise infusion protocols can be free of major cardiopulmonary distress, provided the infusion does not exceed certain threshold rates, which are

different for the first and subsequent infusion steps. Under the conditions of our study these thresholds values were 0.24 and 2.5 mg phospholipid/kg/min for the initial and subsequent infusion periods, respectively.

Features of hemodynamic and thromboxane A2 responses in the 2- and 3-step dose escalation protocols

Fig 3A and B shows the time courses of PAP changes in pigs infused with PLP according to protocols B and C, respectively, along with the plasma TxB2 readings at different times. In keeping with Fig. 2, the mean values of both parameters were higher in "B pigs" compared to "C pigs", however, the SD values were also higher, suggesting greater variation of response in protocol B. The figure also shows clear concordance between the rises and falls of PAP and TxB2, which is consistent with the causal role of TxA2 in liposome-induced pulmonary hypertension in pigs [7].

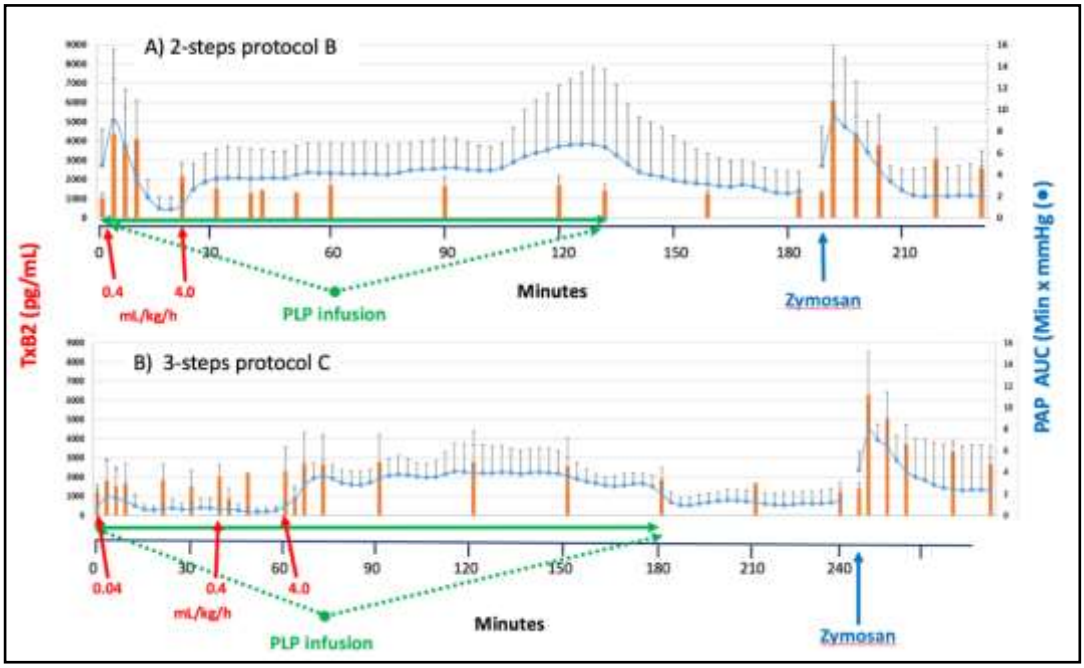

Figure 3. Time course of TxB2 (red bars) and PAP (blue line) changes (Mean ± SD SD) in pigs treated with PLP with the 2-step (B) (n = 9) and 3-step (C, n= 4) infusion protocols. Red arrows point to the time when the infusion was started at the rate specified by the number (in mL/kg/h). Green shows the duration of infusion. Zymosan was administered at 0.1 mg/kg.

Fig. 4 shows the correlation between PAP and TxB2 values in protocol B and C pigs. Consistent with the significant rise and substantial individual variation of PAP in B animals (Table 3), the paired PAP-TxB2 values segregated into two groups; 1/3 (n = 3) of pigs displayed relatively high PAP and TxB2 values that showed significant correlation (Fig 4, upper regression line), while

2/3 (n=6) of the animals showed minor or no changes relative to baseline. This suggests that protocol B represents a borderline in terms of risk for initial pulmonary hypertension, leading to the conclusion that the protocol cannot be considered as reaction-free. In contrast, all 4 of "C pigs" showed minor or no change of initial PAP and TxB2 with no statistical difference relative to baseline. These observations, although in a small number of animals, suggest that the serendipitously tested 8-10-fold reduction of initial (and first step-up) infusion rates that we applied in infusion protocol C versus B minimized the risk of TxB2 release and consequent initial pulmonary hypertension. Thus, under the experimental conditions of this study the infusion parameters in protocol C provided the best administration protocol in terms of risk for hyperacute (within minutes) HSRs. It should be emphasized, however, that this relative "safety" applies only to the first, initial reaction, as the cause and biological relevance of gradually developing 2-3-fold constant rise of TxB2 and pulmonary hypertension after 1 h infusion remains to be established.

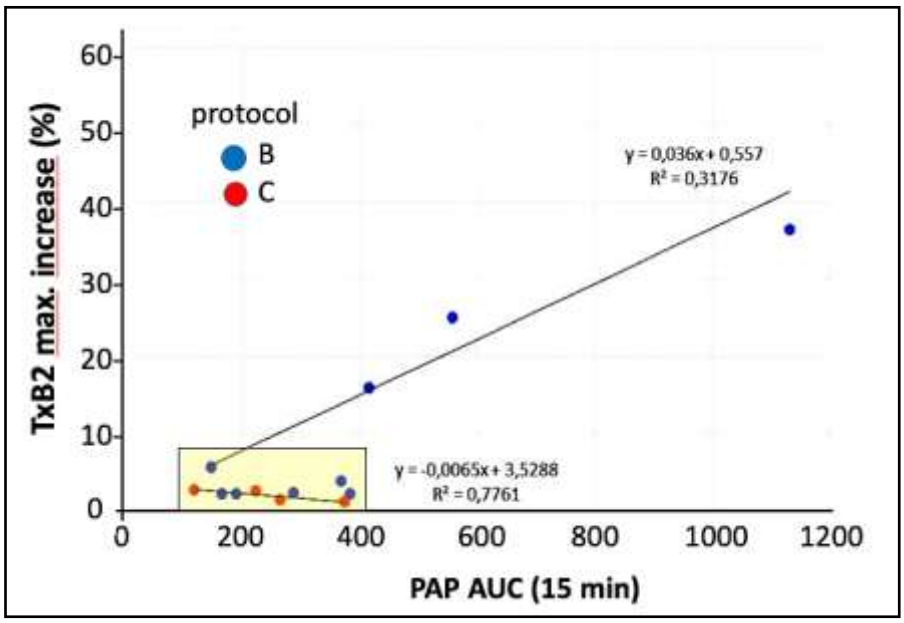

Figure 4. Correlation between the first 15 min PAP AUC values and maximal increases in the TxB2 blood concentration (percentage of 0' value) in 4/9 animals treated with protocol B. The values in the shaded rectangle represent small, biologically irrelevant changes.

Discussion

Infusion reactions have been observed ever since infusion therapy has been implemented in modern medicine, yet even today it represents a safety issue for many drugs and drug candidates [11-13]. Their mechanism is poorly understood, but it is clear that multiple immunological

pathways are involved, the relative contributions of which may vary from case to case. One pathway gaining recent attention involves complement activation, a possible trigger mechanism of HSRs to nanomedicines and biologicals [14]. Complement activation can trigger HSRs by at least two pathways, via release of anaphylatoxins [15], and also via opsonization of the trigger agent enhancing its binding to (with or without uptake by) macrophages or other complement-receptor containing allergy mediating cells, which respond with secretion of bioactive mediators. Both phenomena have been observed in pigs [16, 17]. However, a portion of acute physiological changes corresponding to HSR may be triggered independent of complement activation, such as the liposome-induced acute hypertension in mice [18]. Thus, while a variety of symptoms can be generated by one single "hit" on allergy mediating cells, the broadest vision HSRs raises the possibility of two or more "hits", suggesting that the variety of symptoms is due to the individual variation of these "hits" [19].

The current, standard approach of preventing HSRs is premedication of the patient with steroids, antihistamines and other anti-inflammatory drugs, and administration of the drug in slow infusion. There is no doubt that these measures are effective, without them a large number of drugs could not be used in infusion therapy. However, these methods are not full-proof, either, as occasionally, despite all attention and effort, severe HSRs occur and cause death. Just focusing on PEGylated pharmaceuticals, over the past few years three were withdrawn from clinical use partly because of severe HSRs: PEGylated EPO-mimetic peptide (*Peginesatide*, *Omontys*®) [20], PEGylated urate oxidase (*Pegloticase*, *Krystexxa*®) [21, 22] and a PEGylated IXa blocker RNA aptamer *(Pegnivacogin*, *Revolixys*®) [23]. These facts lend importance to studies that try to understand these reactions and develop new ways of their prevention.

The above goals can most efficiently be achieved by using appropriate animal models. The pros and cons of the pig model was recently reviewed [1], and one of the conclusions was that the high sensitivity of the model makes it an efficient preclinical screening test for anaphylactoid reactogenicity of nanoparticle-based drugs and other agents [1]. It was also emphasized that it is a disease model, that of hypersensitive man, and that it can be used both for hazard identification and mitigation [1]. Nevertheless, the pig model was recently questioned on the basis that the prevalence of HSRs does not reproduce the average human reaction rate, the mechanisms are different and that the cardiovascular changes represent a "global response", i.e., the model has no capability to differentiate among nanoparticles in terms of reactogenicity [24-26]. Hence, it was judged as "misleading" that "should not be advertently promoted" for safety evaluation [24-26]. However, these arguments were contradicted by a study spearheaded by the lead author of the above critical

reviews [27], showing that the PAP response in the same pig model used here can quantify and differentiate the reactogenicity of polystyrene nanoparticles on the basis of their shape. Thus -it was concluded-, changing the shape of nanoparticles represents a new strategy for combatting HSRs [27]. In fact, a large number of research studies provide evidence for the utility of the model to predict acute immune reactivity [28-33], and there is also example for the use of the model in the pharmaceutical industry, in developing safe administration protocols for nucleotide-containing lipid nanoparticles, such as the first FDA-approved gene therapeutic agent, Patisiran (Onpattro®) [34].

The present study represents an addition to the list of studies utilizing the pig model for risk identification and mitigation, measuring the CARPAgenicity of PLP and developing the safest infusion protocol for its administration. We used two sensitive endpoints, PAP and TxB2 as measures of HSRs, which were previously shown to correlate with each other [7]. The paralleling rises of these variables and confirmation of their correlation (Fig 4) in the present study confirmed the consistency of the model and the concept of cause-effect relationship between TxB2 secretion and pulmonary hypertension in the efferent arm of HSRs [7, 8].

Our finding that bolus injection of PLP in pigs caused very similar, tachyphylactic (self-limiting) HSR as bolus injection of pegylated liposomal doxorubicin (Doxil) suggests that the differences between the two liposome formulations in terms of encapsulated drug and bilayer composition are not critical for triggering reactogenicity. The common denominator that may control the reaction is the pegylated liposome surface, i.e. the phospholipid bilayer coated with ~5% 2K-PEG.

Furthermore, the finding that the slowest, 3-step dose escalation protocol was the safest in terms of cardiovascular reactivity is in line with the well-known reaction-lessening effect of slow infusion of reactogenic nano-biopharmaceuticals. For the case of PLP, an initial infusion rate of 0.03 ml/kg/h over 40 min turned out to be reaction-free as opposed to 0.24 ml/kg/h over 40 min, suggesting that the no observed adverse effect level (NOAEL) is in the 0.03-0.24 ml/kg/h range, at least under the conditions of this study. Whether or not these numbers hold up for other nanomedicines in pigs, or in man, remains to be established in further studies.

The observation that intra-liposomal prednisolone did not inhibit the rise of PAP or TxB2 at times when macrophages or other allergy mediating cells might have taken up PLP during the course of infusion (e.g. the reactions to zymosan) suggests that the immune suppressive effect of prednisolone is not effective against PLP-induced HSR, at least within 2-3 h under the conditions of this study.

As for the mechanism by which slow infusion might mitigate the reactogenicity of liposomal and other nanoparticulate drugs, the "anaphylatoxin balance concept" [35] represents one possible explanation. According to this theory, the blood level of anaphylatoxins, C3a and C5a, is determined by their generation via C activation and clearance by cellular uptake and metabolism by carboxypeptidases [36]. If massive anaphylatoxin formation exceeds its clearance, which is much slower, its blood level may rapidly spike to reach a threshold where the allergy mediating cells release their mediators. In contrast, slow formation of anaphylatoxins during slow infusion may be coped with by clearance, keeping the concentration of anaphylatoxins below the HSR threshold.

In summary, the present data, together with numerous other studies provide support for using the porcine CARPA model for assessing the reactogenicity of PEGylated nanomedicines, such as PLP, and for developing safe infusion protocols for their administration. These protocols may vary for different PEGylated and non-PEGylated nanoparticles with different reactogenicities, and the pig model might help in fine-tuning the optimal parameters. Nevertheless, further studies are needed to establish the concordance of pig and human symptoms of HSRs to different nanoparticles, and thus validate extended use of the model in preclinical safety testing.

Acknowledgments

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References

1. J. Szebeni, P. Bedőcs, L. Dézsi, R. Urbanics, A porcine model of complement activation-related pseudoallergy to nano-pharmaceuticals: Pros and cons of translation to a preclinical safety test, Prec. Nanomed. , 1 (2018) 63-73.
2. J. Szebeni, B. Baranyi, S. Savay, M. Bodo, D.S. Morse, M. Basta, G.L. S1tahl, R. Bunger, C.R. Alving, Liposome-induced pulmonary hypertension: Properties and mechanism of a complement-mediated pseudoallergic reaction, Am. J. Physiol., 279 (2000) H1319-H1328.

3. G. Calogiuri, M.T. Ventura, L. Mason, A. Valacca, R. Buquicchio, N. Cassano, G.A. Vena, Hypersensitivity reactions to last generation chimeric, humanized [correction of umanized] and human recombinant monoclonal antibodies for therapeutic use, Curr Pharm Des, 14 (2008) 2883-2891.
4. J.M. van den Hoven, R. Nemes, J.M. Metselaar, B. Nuijen, J.H. Beijnen, G. Storm, J. Szebeni, Complement activation by PEGylated liposomes containing prednisolone, Eur J Pharm Sci, 49 (2013) 265-271.
5. F.M. van der Valk, D.F. van Wijk, M.E. Lobatto, H.J. Verberne, G. Storm, M.C. Willems, D.A. Legemate, A.J. Nederveen, C. Calcagno, V. Mani, S. Ramachandran, M.P. Paridaans, M.J. Otten, G.M. Dallinga-Thie, Z.A. Fayad, M. Nieuwdorp, D.M. Schulte, J.M. Metselaar, W.J. Mulder, E.S. Stroes, Prednisolone-containing liposomes accumulate in human atherosclerotic macrophages upon intravenous administration, Nanomedicine, 11 (2015) 1039-1046.
6. F.M. van der Valk, D.M. Schulte, S. Meiler, J. Tang, K.H. Zheng, J. Van den Bossche, T. Seijkens, M. Laudes, M. de Winther, E. Lutgens, A. Alaarg, J.M. Metselaar, G.M. Dallinga-Thie, W.J. Mulder, E.S. Stroes, A.A. Hamers, Liposomal prednisolone promotes macrophage lipotoxicity in experimental atherosclerosis, Nanomedicine, 12 (2016) 1463-1470.
7. J. Szebeni, J.L. Fontana, N.M. Wassef, P.D. Mongan, D.S. Morse, D.E. Dobbins, G.L. Stahl, R. Bunger, C.R. Alving, 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, 99 (1999) 2302-2309.
8. R. Urbanics, J. Szebeni, Lessons learned from the porcine CARPA model: constant and variable responses to different nanomedicines and administration protocols, Eur J Nanomedicine, 7 (2015) 219-231.
9. J. Szebeni, P. Bedocs, Z. Rozsnyay, Z. Weiszhar, R. Urbanics, L. Rosivall, R. Cohen, O. Garbuzenko, G. Bathori, M. Toth, R. Bunger, Y. Barenholz, Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome, Nanomedicine, 8 (2012) 176-184.
10. J. Szebeni, P. Bedocs, R. Urbanics, R. Bunger, L. Rosivall, M. Toth, Y. Barenholz, Prevention of infusion reactions to PEGylated liposomal doxorubicin via tachyphylaxis induction by placebo vesicles: a porcine model, J Control Release, 160 (2012) 382-387.

11. A. Cheifetz, L. Mayer, Monoclonal antibodies, immunogenicity, and associated infusion reactions, Mt Sinai J Med., 72 (2005) 250-256.
12. L. Mayer, Y. Young, Infusion reactions and their management, Gastroenterol. Clin. North Am., 35 (2006) 857-866.
13. J. Szebeni, D. Simberg, A. Gonzalez-Fernandez, Y. Barenholz, M.A. Dobrovolskaia, Roadmap and strategy for overcoming infusion reactions to nanomedicines, Nat Nanotechnol, (2018).
14. J. Szebeni, Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals, Mol. Immunol., 61 (2014) 163-173.
15. T.E. Hugli, N.P. Stimler, C. Gerard, K.E. Moon, Possible role of serum anaphylatoxins in hypersensitivity reactions, Int Arch Allergy Appl Immunol, 66 Suppl 1 (1981) 113-120.
16. J. Szebeni, L. Baranyi, S. Savay, M. Bodo, J. Milosevits, C.R. Alving, R. Bunger, 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, 290 (2006) H1050-1058.
17. T. Meszaros, G.T. Kozma, T. Shimizu, K. Miyahara, K. Turjeman, T. Ishida, Y. Barenholz, R. Urbanics, J. Szebeni, Involvement of complement activation in the pulmonary vasoactivity of polystyrene nanoparticles in pigs: unique surface properties underlying alternative pathway activation and instant opsonization, Int J Nanomedicine, 13 (2018) 6345-6357.
18. E. Orfi, T. Meszaros, M. Hennies, T. Fulop, L. Dezsi, A. Nardocci, L. Rosivall, P. Hamar, B.W. Neun, M.A. Dobrovolskaia, J. Szebeni, G. Szenasi, Acute physiological changes caused by complement activators and amphotericin B-containing liposomes in mice, Int J Nanomedicine, 14 (2019) 1563-1573.
19. J. Szebeni, Mechanism of nanoparticle-induced hypersensitivity in pigs: complement or not complement?, Drug Discov Today, 23 (2018) 487-492.
20. T. Hermanson, C.L. Bennett, I.C. Macdougall, Peginesatide for the treatment of anemia due to chronic kidney disease - an unfulfilled promise, Expert Opin Drug Saf, 15 (2016) 1421-1426.
21. Krystexxa® (pegloticase injection), for intravenous infusion: Prescribing Information Revised 2018, https://hznp.azureedge.net/public/KRYSTEXXA_Prescribing_Information.pdf, (2018).
22. Krystexxa: Withdrawal of the marketing authorisation in the European Union, EMA/498114/2016, https://www.ema.europa.eu/en/documents/public-statement/public-

statement-krystexxa-withdrawal-marketing-authorisation-european-union_en.pdf (2016) 21 July 2016.

23. T.J. Povsic, J.P. Vavalle, L.H. Aberle, J.D. Kasprzak, M.G. Cohen, R. Mehran, C. Bode, C.E. Buller, G. Montalescot, J.H. Cornel, A. Rynkiewicz, M.E. Ring, U. Zeymer, M. Natarajan, N. Delarche, S.L. Zelenkofske, R.C. Becker, J.H. Alexander, R. Investigators, A Phase 2, randomized, partially blinded, active-controlled study assessing the efficacy and safety of variable anticoagulation reversal using the REG1 system in patients with acute coronary syndromes: results of the RADAR trial, Eur Heart J, 34 (2013) 2481-2489.
24. S.M. Moghimi, Nanomedicine safety in preclinical and clinical development: focus on idiosyncratic injection/infusion reactions, Drug Discov Today, 23 (2018) 1034-1042.
25. S.M. Moghimi, D. Simberg, Translational gaps in animal models of human infusion reactions to nanomedicines, Nanomedicine (Lond), 13 (2018) 973-975.
26. S.M. Moghimi, D. Simberg, T. Skotland, A. Yaghmur, C. Hunter, The Interplay between Blood Proteins, Complement, and Macrophages on Nanomedicine Performance and Responses, J Pharmacol Exp Ther, (2019).
27. P.P. Wibroe, A.C. Anselmo, P.H. Nilsson, A. Sarode, V. Gupta, R. Urbanics, J. Szebeni, A.C. Hunter, S. Mitragotri, T.E. Mollnes, S.M. Moghimi, Bypassing adverse injection reactions to nanoparticles through shape modification and attachment to erythrocytes, Nat Nanotechnol, 12 (2017) 589-594.
28. H. Epstein-Barash, D. Gutman, E. Markovsky, G. Mishan-Eisenberg, N. Koroukhov, J. Szebeni, G. Golomb, Physicochemical parameters affecting liposomal bisphosphonates bioactivity for restenosis therapy: internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death, Journal of Controlled Release, 146 (2010) 182-195.
29. M. Buscema, S. Matviykiv, T. Meszaros, G. Gerganova, A. Weinberger, U. Mettal, D. Mueller, F. Neuhaus, E. Stalder, T. Ishikawa, R. Urbanics, T. Saxer, T. Pfohl, J. Szebeni, A. Zumbuehl, B. Muller, Immunological response to nitroglycerin-loaded shear-responsive liposomes in vitro and in vivo, J Control Release, 264 (2017) 14-23.
30. H. Unterweger, C. Janko, M. Schwarz, L. Dezsi, R. Urbanics, J. Matuszak, E. Orfi, T. Fulop, T. Bauerle, J. Szebeni, C. Journe, A.R. Boccaccini, C. Alexiou, S. Lyer, I. Cicha, Non-immunogenic dextran-coated superparamagnetic iron oxide nanoparticles: a biocompatible, size-tunable contrast agent for magnetic resonance imaging, Int J Nanomedicine, 12 (2017) 5223-5238.

31. H. Unterweger, L. Dezsi, J. Matuszak, C. Janko, M. Poettler, J. Jordan, T. Bauerle, J. Szebeni, T. Fey, A.R. Boccaccini, C. Alexiou, I. Cicha, Dextran-coated superparamagnetic iron oxide nanoparticles for magnetic resonance imaging: evaluation of size-dependent imaging properties, storage stability and safety, Int J Nanomedicine, 13 (2018) 1899-1915.
32. J. Matuszak, J. Baumgartner, J. Zaloga, M. Juenet, A.E. da Silva, D. Franke, G. Almer, I. Texier, D. Faivre, J.M. Metselaar, F.P. Navarro, C. Chauvierre, R. Prassl, L. Dezsi, R. Urbanics, C. Alexiou, H. Mangge, J. Szebeni, D. Letourneur, I. Cicha, Nanoparticles for intravascular applications: physicochemical characterization and cytotoxicity testing, Nanomedicine (Lond), 11 (2016) 597-616.
33. I. Cicha, C. Chauvierre, I. Texier, C. Cabella, J.M. Metselaar, J. Szebeni, L. Dezsi, C. Alexiou, F. Rouzet, G. Storm, E. Stroes, D. Bruce, N. MacRitchie, P. Maffia, D. Letourneur, From design to the clinic: practical guidelines for translating cardiovascular nanomedicine, Cardiovasc Res, 114 (2018) 1714-1727.
34. P. Kasperovic, Dosages and methods for delivering lipid formulated nucleic acid molecules, US patent applicationsd #20160122759, (2016).
35. J. Szebeni, S. Fishbane, M. Hedenus, S. Howaldt, F. Locatelli, S. Patni, D. Rampton, G. Weiss, J. Folkersen, Hypersensitivity to intravenous iron: classification, terminology, mechanisms and management, Br J Pharmacol, 172 (2015) 5025-5036.
36. W.D. Campbell, E. Lazoura, N. Okada, H. Okada, Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N, Microbiol Immunol, 46 (2002) 131-134.

3

Complement activation in vitro and reactogenicity of low-molecular weight dextran-coated SPIONs in the pig CARPA model: Correlation with physicochemical features and clinical information

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Abbreviations:

C, complement
CARPA, complement activation-related pseudoallergy HSR, hypersensitivity reaction
MRI, magnetic resonance imaging NTA, nanoparticle tracking analysis PDI, polydispersity index
PEG, polyethylene glycol
SPION, superparamagnetic iron-oxide nanoparticle
USPION, ultra-small SPION

Abstract

The unique magnetic properties of superparamagnetic iron oxide nanoparticles (SPIONs) have

led to their increasing use in drug delivery and imaging applications. Some polymer-coated SPIONs, however, share with many other nanoparticles the potential of causing hypersensitivity reactions known as complement (C) activation- related pseudoallergy (CARPA). In order to explore the roles of iron core composition and particle surface coating in SPION-induced CARPA, we measured C activation by 6 different SPIONs in a human serum that is known to react to nanoparticles (NPs) with strong C activation. Remarkably, only the Fe^{2+} nucleated, carboxymethyldextran-coated (ferucarbotran, Resosvist®) and Fe^{3+}/Fe^{2+} nucleated, dextran-coated (ferumoxtran-10, Sinerem®) SPIONs caused significant C activation, while the Fe_3O_4-nucleated acid, phosphatidylcholine, starch and chitosan-coated SPIONs had no such effect. Focusing on Resovist and Sinerem, we found Sinerem to be a stronger activator of C than Resovist, although the individual variation in 15 different human sera was substantial. Further analysis of C activation by Sinerem indicated biphasic dose dependence and significant production of C split product Bb but not C4d, attesting to alternative pathway C activation only at low doses. Consistent with the strong C activation by Sinerem and previous reports of HSRs in man, injection of Sinerem in a pig led to dose-dependent CARPA, while Resovist was reaction-free. Using nanoparticle tracking analysis, it was further determined that Sinerem, but not Resovist, possessed a multimodal size distribution and significant fraction of aggregates – factors which are known to promote C activation and CARPA. Taken together, our findings offer physicochemical insight into how key compositional factors and nanoparticle size distribution affect SPION-induced CARPA, and leveraging such knowledge could lead to the development of SPIONs with improved safety profiles.

Keywords: complement, hypersensitivity reactions, iron, anaphylaxis, CARPA, anaphylatoxins, MRI, imaging, nanoparticles, nanomedicines, immune toxicity

Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs), state-of-art representatives of clinically useful nanoparticles (NPs), have been used as contrast agents for magnetic resonance imaging (MRI) over the past decade [1-5]. They are in the 10-100 nm range (up to 30 nm in the case of USPIONs) and contain a γ-Fe_2O_3 (maghemite), Fe_3O_4 (magnetite) or α-Fe_2O_3 (hermatite) core and a hydrophilic surface coating made from a variety of polymers, including dextran, carboxydextran, chitosan, phospholipids, PEG and starch. The iron core lends these particles "superparamagnetism", in essence external magnetic field-controllable magnetism that enables

these particles to be utilized for imaging, molecular structure analysis or to the benefit of drug delivery, gene therapy and many other potential applications [6-8].

Among the unsolved challenges of the clinical application of these agents, iron- containing drugs and contrast media can cause hypersensitivity reactions (HSRs). The symptoms of HSRs reported for reactogenic iron-compounds include dyspnea, chest/back pain, hypo/hypertension, fever, flushing, rash and panic, that are also typical symptoms of the HSRs to liposomal and micellar drugs, biological therapeutics, radiocontrast agents, enzymes, PEGylated proteins and many other "nano-bio-pharmaceuticals". These reactions were proposed to be due, at least in part, to activation of the complement (C) system, leading to the term "C activation-related pseudoallergy (CARPA) [9-13]. The worst outcome of CARPA is anaphylaxis with occasional death, which contributed to market withdrawal of ferumoxide (Feridex/Endorem®) [14], ferumoxytol (Feraheme®/Rienso®) [15] and ferumoxtran (Ferumoxtran-10/Sinerem/Combidex) [16].

As implied in its name, the essence of CARPA is the capability of the drug or agent to cause C activation. Such activation has been shown for many of the above listed CARPAgenic drugs [10] including 20-kD dextran-coated SPIONs called "nanoworms" [19-23]. These $FeCl_2/FeCl_3$ precipitates with multiple crystalline cores were shown to activate all 3 (classical, alternative and lectin) pathways in human serum [23], highlighting the redundancy of C activating triggers. Likewise, a recent study reported C activation by iron dextran and ferric carboxymaltose both in vitro and in vivo in the blood of healthy volunteers and hemodialysis patients, leading to the conclusion that HSRs to these drugs could represent CARPA [24]. In fact, the concept that CARPA may underlie many HSRs to iron-containing drugs is gaining increasing attention [25], motivating further exploration of the C reactivity and CARPAgenic activity of SPIONs.

Accordingly, the first goal of our study was to measure C activation by different SPIONs in vitro, and to identify the structural factors responsible for such activation, if it exists. Having found that only the dextran coat caused C activation, we went further to compare different dextran-coated SPIONs, including ferumoxtran (Sinerem®, Combidex) and ferucarbotran (Resovist®) for which information on the HSRs they caused in patients allowed us to correlate the in vitro and animal data obtained in this study with the past human observations.

Materials and Methods

Materials:

Superparamagnetic iron-oxide nanoparticles were obtained from Chemicell GmbH (Germany) and Nano4Imaging (Germany). Quidel's SC5b-9, Bb and C4d C ELISAs were obtained from TECOmedical NL (The Netherlands). Zymosan was obtained from Sigma-Aldrich. Mixed breed male Yorkshire/Hungarian White Landrace pigs (2-3 months old, 18-22 kg) were obtained from the Animal Breeding and Nutrition Research Institute, Herceghalom, Hungary. Resovist® and Sinerem® were kindly offered from Nano4Imaging.

Methods:

Measurement of complement activation in human serum in vitro

Sera from healthy volunteers were incubated with the tested polymers and SPIONs for 30 min at 37°C at a serum/nanoparticle volume ratio of 4:1, duplicate tubes. The iron content of particles was matched. Incubation was stopped by diluting the samples with EDTA containing sample diluent from the ELISA kits, and aliquots from these diluted sera were subjected to measuring SC5b-9, Bb and C4d as pathway specific markers of C activation [26, 27]. In addition we also applied a modified hemolytic C (CH50) assay, as described earlier [28]. In short, were incubated in the sera at 37°C for 30 min followed by a 10-fold dilution in PBS. Aliquots from these diluted sera were incubated with sensitized SRBCs for 10 min at 37°C. The reaction was stopped by centrifugation of the cells at 4oC and measurement of released hemoglobin at 541nm.

In vivo test of CARPA in pigs

Resovist and Sinerem were tested for CARPA in pigs, according to a procedure described previously [13, 29-31]. In brief, animals were sedated with Calypsol/Xilazine and then anesthetized with isoflurane (2–3% in O_2). Intubation was performed with endotracheal tubes to maintain free airways, and to enable controlled ventilation if necessary. The animals were breathing spontaneously during the experiments. In order to measure the pulmonary arterial blood pressure (PAP), a Swan–Ganz catheter (Teleflex Medical, Research Triangle Park, NC, USA) was placed to the pulmonary artery wedge, for the measurement of pulmonary arterial pressure

(PAP). Additional catheters were placed into the femoral artery to record the systemic arterial pressure (SAP). The left femoral vein was cannulated for blood sampling, and the external jugular vein for the administration of SPIONs. The hemodynamic, EKG and respiratory parameters were measured continuously, while blood cell counts, blood analytes and biomarkers (inflammatory and vasoactive mediators) were measured at predetermined times, usually in 10–20 min intervals.

Characterization of nanoparticles

Size and zeta potential measurements

The mean diameter and size distribution (polydispersity index, PDI) of SPIONs were determined by dynamic light scattering (DLS) with Malvern ALV CGS-3 system (Malvern instruments Ltd., Malvern,Worcestershire, United Kingdom) with a scattering angle of 90° at 25 °C. Samples were diluted 200 times using pure 18.2 MΩ cm distilled (Milli-Q, Millipore, Molsheim, France) water before measurement.

The zeta-potential was measured by laser Doppler electrophoresis using Zetasizer Nano-Z (Malvern instruments Ltd., Malvern,Worcestershire, United Kingdom). The nanoparticles were diluted approximately 100 times in 10 mM HEPES (pH 7.4) before measurement.

Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis (NTA) was conducted with a Nanosight LM10 instrument. The particles were diluted to a concentration of 5 μg/ml in Milli-Q-treated water. A 405 nm laser was used to illuminate and cause Rayleigh scattering by the particles, which was visualized by optical microscope (20x magnification). The time- resolved Brownian motion of individual particles was recorded by camera for a time period of 60s at a rate of 25 frames per second, enabling the calculation of the hydrodynamic diameter of individual particles by the two-dimensional Stokes-Einstein equation and construction of a number-weighted size histogram. Video capture and data analysis parameters were controlled using the NTA 3.1 software program. All measurements were carried out under room temperature of 24^{o}C.

Statistical analysis

Comparisons of multiple groups were made by ANOVA followed by Tukey's Multiple Comparison Test, with $P<0.05$ taken a significant difference.

Results and Discussion

Physicochemical characterization of iron-NPs

Table 1 shows some physicochemical properties and other specifics of iron NPs used in this study. The diameters specified represent the mean values obtained by DLS (See Methods).

Core	Coating/vehicle	Generic name	Trade Name	Diameter (nm) / PDI	Zeta Pot. (mV)	Manufacturer
Experimental						
Fe_3O_4	Citric acid-	-	fluidMAG-CT	140 / 0,2	-35,9	Chemicell GmbH
Fe_3O_4	Phosphatidyl-choline	-	fluidMAG-Lipid	189 / 0,3	-33	Chemicell GmbH
Fe_3O_4	Starch	-	fluidMAG-D	40 / 0,1	-9,7	Chemicell GmbH
Fe_3O_4	Chitosan	-	fluidMAG-Chitosan	68 / 0,2	4,7	Chemicell GmbH
Clinically used (discontinued)						
Fe2+	Carboxydextran	ferucarbotran	Resovist® (EU) Cliavist® (US)	60.6 / 0,2	-26,4	Bayer Shering Pharma AG
Fe3+/Fe2+	Dextran	ferumoxtran-10	(Sinerem®, EU) Combidex® (US)	50.7 / 0,3	-13,8	Guerbet, Advanced Magnetics

Table 1. Physicochemical properties of SPIONs. Abbreviations in the Table: PDI, polydispersity index, Pot, potential;

Complement activation of different SPIONs in a sensitive human serum

Previous studies on nanomedicine-induced C activation in normal (healthy) human sera in vitro and patients in vivo showed substantial individual variation, with 1-10% of humans being highly reactive for specific nanodrugs [32-39]. This variation has not been understood to date, thus, as a useful step in experiments comparing C activation by NPs, we have chosen a "reactive donor serum", i.e., a serum which gave strong C activation in previous experiments (mainly by liposomal doxorubicin, Doxil [36]. Fig. 1A-C shows C activation in this preselected reactive serum by SPIONs with different cores and surface coatings at 3 different concentrations: 0.1, 0.5 and 10 mg/mL iron (panels A-C, respectively). Chitosan and phosphatidylcholine coatings were reaction free at all doses, starch and carboxymethyldextran caused minor C activation, while dextran caused massive activation. Moreover, the C activating effect effect of dextran showed biphasic

dose-dependence: 0.1 and 0.5 mg/mL were stimulatory, while 10 mg/mL was inhibitory. Indications of an inverse dose-effect relationship between dextran and C activation was also seen in another independent experiment when free dextran was incubated with 3 normal human sera (NHS) (Fig 1D), suggesting that concentration is a critical factor in the immune activity of this polymer.

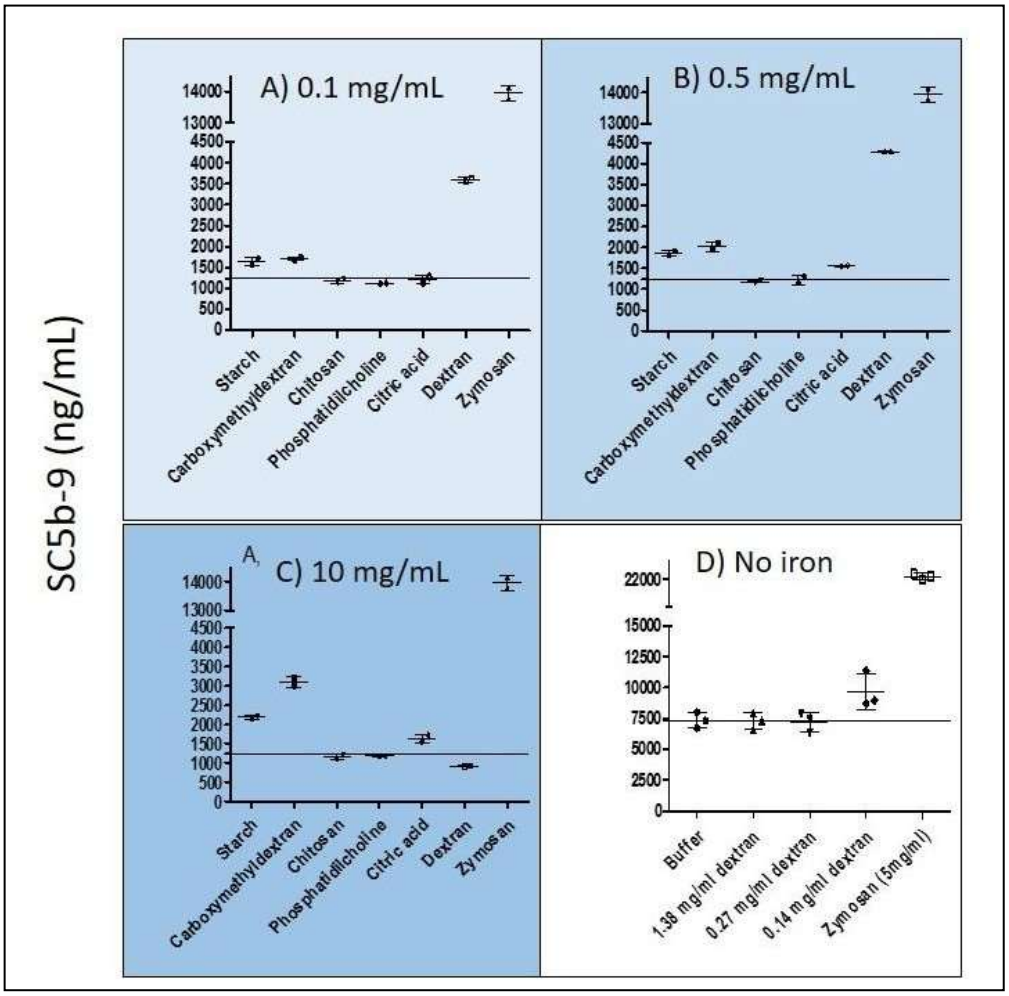

Figure 1. Complement activation by different SPIONs in NHS after incubation for 30 min at 37 C. The horizontal line: SC5b-9 in PBS (baseline). The positive control zymosan was applied at 5 mg/ml. The error bars represent SD of duplicate measurements (n=2).

Taken together, these data provide evidence that polymer coatings in general, and dextran coatings in particular, can make SPIONs become potent C activators in certain sensitive individuals.

Complement activation by Resovist and Sinerem in normal human sera: individual variation

Focusing on the stimulatory effect of low doses of carboxymethyl dextran (Resovist) and dextran

(Sinerem), we next investigated the individual variation of C activation by these iron-NPs in NHS. As shown in Fig 2A, incubation of Sinerem with 5 different NHS led to greater rise of SC5b-9 over baseline (up to ≈ 20-fold rise) than that caused by Resovist (Fig 2B, up to ≈ 5-fold rise), and the number of reactive patients was also higher in the former group (5/5 vs. 3/5).

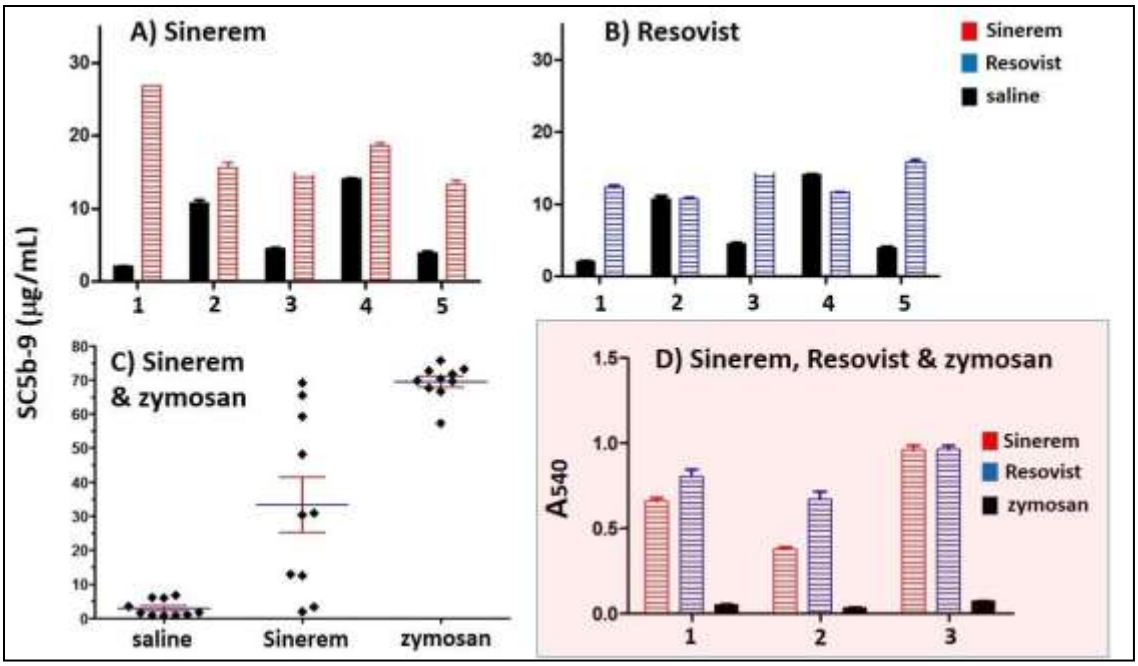

Figure 2. Complement activation by Sinerem and Resovist under different experimental conditions. In A, 5 different NHS were incubated with 0.25 mg/mL Sinerem (iron), and in B, the same dose of Resovist was applied in the same 5 sera. C, a different set of 10 NHS was incubated with 0.25 mg/mL Sinerem or 0.1 mg/ml zymosan. ANOVA followed by Tukey's Multiple Comparison Test showed the 3 groups significantly differing from each other ($P<0.05$). Panel D shows the results of a hemolytic assay, testing Sinerem, Resovist and zymosan at the same levels as in A-C. The sheep red cell assay applied was described in the Methods. Bars are mean ± SD for duplicate measurements in each sera.

Questioning the inter-experimental variation of the individual variation of Sinerem- induced C activation in NHS, we tested yet another independent series of different sera and repeated the same experiment as shown in Fig 2A, using this time 10 sera and zymosan as positive control. As shown in Fig 2 C, the range and variation this time was greater than in Panel A, but the basic message is the same: Sinerem activates human C with substantial individual variation. Based on this series, 8 of 10 (80%) of sera showed significant reactivity against Sinerem, and 3 in 10 (20-30%) showed activation comparable to that caused by 0.1 mg/mL zymosan.

SC5b-9 is a soluble end-product of C activation, whose individual variation may have reasons independent of the central reaction, i.e. C3 conversion and subsequent cascadic formation of the terminal complex (C5b-9). For this reason, we also evaluated the effects of Sinerem and Resovist

on the whole C cascade by using a modified hemolytic assay which measured the consumption of all C proteins that are involved in hemolysis (hemolytic C). Figure 2D shows greater consumption of hemolytic C by Sinerem than Resovist in 2 of the 3 tested sera, and this finding is consistent with the conclusions drawn from the SC5b-9 ELISA. Thus, the differential effects of tested SPIONs apply to the whole C cascade.

Pathway of complement activation by Sinerem

In order to determine the pathway of C activation by Sinerem we incubated 3 different NHS with Sinerem and measured the production of SC5b-9, C4d and Bb, which are specific markers of the terminal, classical and alternative pathways. The significant and comparable elevations of SC5b-9 and Bb (Figure 3) clearly indicate the involvement of alternative pathway activation, with no, or negligible operation of the classical pathway.

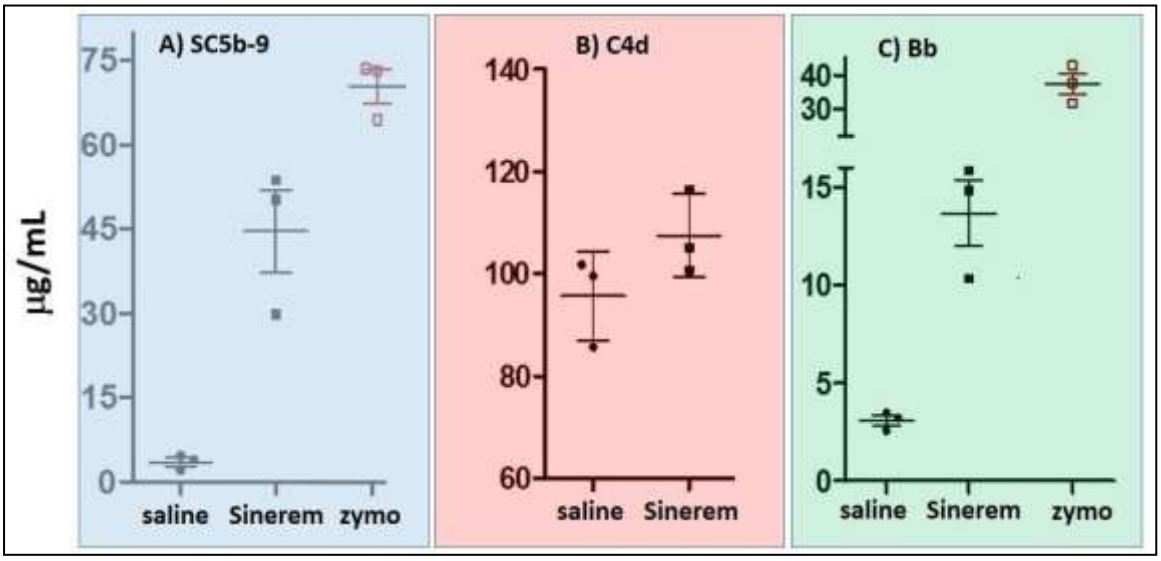

Figure 3. Pathway of C activation by Sinerem tested in 3 NHS. Panels A-C show the results of SC5b-9, C4d abd Bb ELISA performed in 3 NHS. Sinerem and zymosan were applied at 0.25 and 0.1 mg/mL, respectively. Bars are mean ± SD for duplicate measurements in each sera.

CARPAgenic activity of Resovist and Sinerem in pigs

The above in vitro data indicating slight but significant quantitative differences in C activation by Resovist and Sinerem led to a critical question: do these differences entail differential biological responses in an animal model of CARPA? Or more broadly: can in vitro C measurements be

translated to the clinics in predicting HSRs?

To address this question, we used the porcine CARPA model, which is known to be a sensitive quantitative assay of the acute immune reactivity of NPs manifested in anaphylactoid reations [13, 29-32]. We injected Resovist and Sinerem in consecutive boluses at 0.1 and 0.5 mg/kg iron and traced the changes of pulmonary arterial pressure (PAP), systemic arterial pressure (SAP) and heart rate (HR) as hemodynamic endpoints of CARPA. Before these injections, a bolus of saline served as a negative (volume) control, while at the end of the experiment, zymosan served as a positive control, testifying to the intactness of the pigs' cardiovascular reactivity. As shown in Figure 4, Resovist did not cause any changes in the measured parameters (Fig 4A), while Sinerem did cause significant dose-dependent changes (Fig 4B). Namely, 0.01 and 0.1 mg/kg doses led to 30-40 and 50-60% rises in both PAP and SAP, respectively, and the response of PAP, as well as the tachycardia, were clearly more expressed at the higher dose (sub- curve area of PAP 3-fold higher, up to 25 BPM tachycardia).

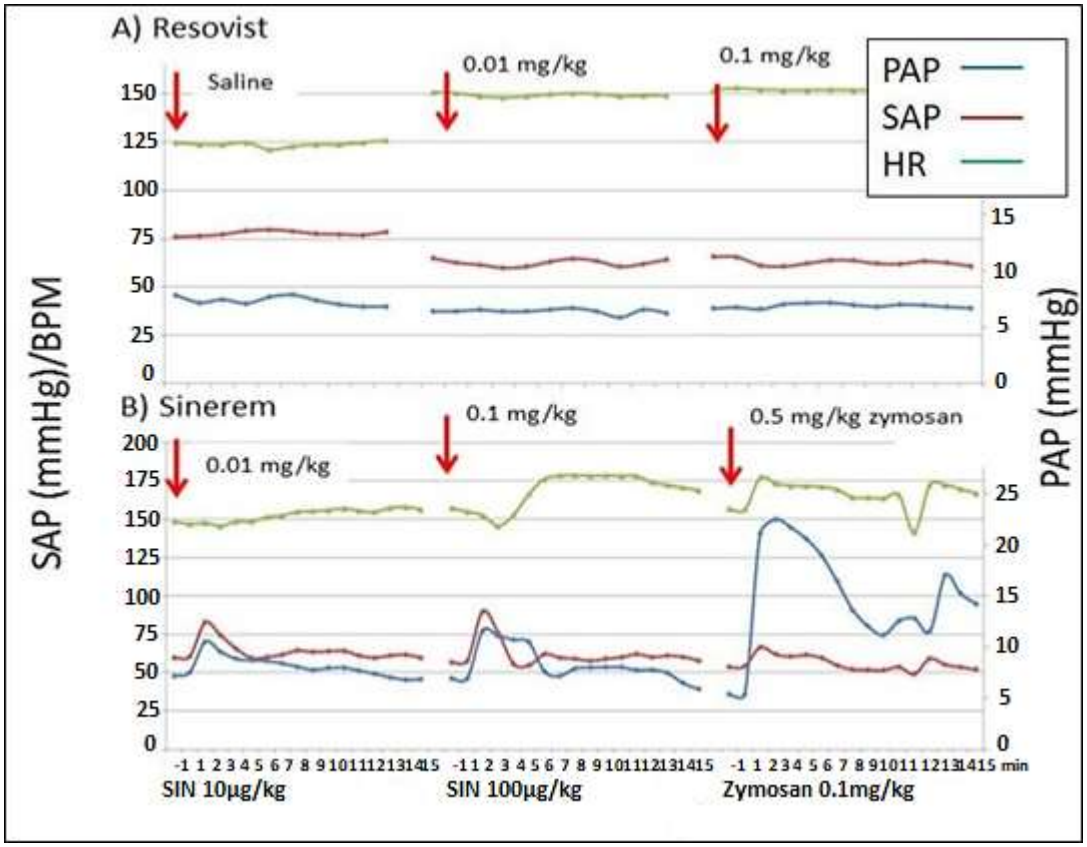

Figure 4. Hemodynamic changes caused by Resovist and Sinerem in pigs. The changes in systemic arterial pressure (SAP, red), pulmonary arterial pressure (PAP, blue) and heart rate (beat per minute, BPM) were followed up to 15 min (X-axis) shown on the x axis.

Nanoparticle tracking analysis of Resovist and Sinerem

In an effort to explain the observed differences between Resovist and Sinerem in C activation and CARPA, we performed additional dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) experiments with these particular SPIONS. As shown in Figure 5A, the DLS technique showed that Resovist NPs ranged in size from around 40 to 120 nm diameter, with 2 peaks between 40-60 and 70-120 nm. In the same sample, the NTA experiment (which measures the concentration, i.e., an absolute number of NPs as a function of hydrodynamic diameter) indicated a peak at about 60 nm with gradual decline of NP number with increasing size until about 360 nm. The minor peaks superimposed on the slope of concentration curve (contour) are indicative of inhomogeneities, most likely aggregates, but these were not as expressed to such an extent as seen for Sinerem (Fig. 5B). For Sinerem, the NTA experiment revealed major peaks around 75, 150 and 225 nm, and the DLS data were also different from that obtained with Resovist, with peaks at about 30 and 80 nm.

While these values agree well with the reported size distribution of SPIONs in general [41], they also reveal quantitative differences between the two studied preparations. Namely, Sinerem had a much larger number of relatively large (> 100 nm) NPs with an inhomogeneous distribution as compared to Resovist. The smaller-size population agrees well with the reported size of Sinerem particles [42], while the larger- size population probably constitutes some aggregates. Of note, NTA has a detection limit around 30 nm and only particles above this minimum size (i.e., in this case, SPION aggregates) can be observed with this technique [43]. Taken together, the NTA measurements suggest that a possible underlying factor behind the increased C activating and CARPAgenic activity of Sinerem vs Resovist is the increased size and inhomogenicity of Sinerem NPs, possibly reflecting the increased presence of aggregates.

In summary, the focus of the present study was to, through a comparative analysis, elucidate physicochemical factors of SPIONs that may cause potentially serious HSR via C activation. Such reactions have been described for the dextran-coated SPIONs, ferumoxides [14] and ferumoxtran-10 (Combidex/(Sinerem) with frequencies in the 2-5% range [16, 44, 45], and this range is also typical of CARPA caused by other nanomedicines and other agents [10]. Recent credit for CARPA being the likely underlying cause of HSRs to iron-containing compounds came from an editorial by Hempel [24]. Regarding SPION reactions. it seems important to refer to a post-

marketing safety review of the FDA in 2005 [46], which gives details of 18 anaphylaxis- related sudden (within 2-30 min) deaths in recipients of various iron dextran preparations including MRI contrast agents. The reported initial symptoms (flushing, shortness of breath, chest/back pain, dizziness, hypo/hypertension, edema, bradyarrhythmia) as well as a cause of death (cardiac arrest) exactly match the symptoms described for the above CARPAgenic drugs [10, 31].

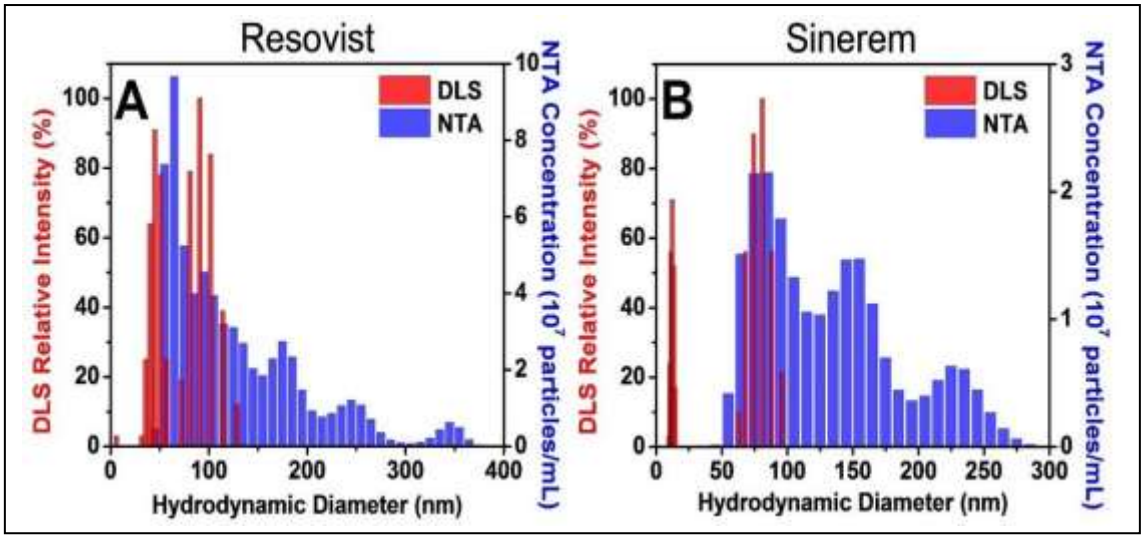

Figure 5. Size distribution of Resovist (A) and Sinerem (B) nanoparticles obtained by DLS and NTA measurements. The data is plotted in the native form: DLS (intensity-weighted; red) and NTA (number- weighted; blue).

The present study confirmed the C activating capability of two dextran-containing SPIONs used in tumor diagnosis; Sinerem and Resovist. Before withdrawing from the market, Sinerem was used for MRI imaging of lymph nodes, while Resovist was a liver specific MRI contrast agent. Our data suggest stronger C activation and stronger in vivo reactivity of Sinerem compared to Resovist, which is consistent with the information that HSRs contributed to the withdrawal from the market of Sinerem [16], but not that of Resovist [17, 18]. We can also correlate the C activation data with at least with one known particle feature known to contribute to C activation and CARPA: inhomogeneity [47]. While the DLS average size info (Table 1) showed no difference, the NTA analysis revealed more expressed inhomogeneity in Sinerem than in Resovist, which could be due to increased amounts of aggregates in the latter preparation. This conclusion highlights the importance of using adequate methods for the physicochemical characterization of nanoparticles, in the present example to quantify inhomogeneity and to detect aggregates.

Acknowledgements

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References

1. R. Jin, B. Lin, D. Li, H. Ai, Superparamagnetic iron oxide nanoparticles for MR imaging and therapy: design considerations and clinical applications, Current opinion in pharmacology, 18 (2014) 18-27.
2. H.J. Kwon, W.H. Shim, G. Cho, H.J. Cho, H.S. Jung, C.K. Lee, Y.S. Lee, J.H. Baek, E.J. Kim, J.Y. Suh, Y.S. Sung, D.C. Woo, Y.R. Kim, J.K. Kim, Simultaneous evaluation of vascular morphology, blood volume and transvascular permeability using SPION-based, dual-contrast MRI: imaging optimization and feasibility test, NMR in biomedicine, 28 (2015) 624-632.
3. X. Ma, A. Gong, B. Chen, J. Zheng, T. Chen, Z. Shen, A. Wu, Exploring a new SPION-based MRI contrast agent with excellent water-dispersibility, high specificity to cancer cells and strong MR imaging efficacy, Colloids and surfaces. B, Biointerfaces, 126 (2015) 44-49.
4. X. Mao, J. Xu, H. Cui, Functional nanoparticles for magnetic resonance imaging, Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology, 8 (2016) 814-841.
5. B. Sivakumar, R.G. Aswathy, R. Romero-Aburto, T. Mitcham, K.A. Mitchel, Y. Nagaoka, R.R. Bouchard, P.M. Ajayan, T. Maekawa, D.N. Sakthikumar, Highly versatile SPION encapsulated PLGA nanoparticles as photothermal ablators of cancer cells and as multimodal imaging agents, Biomaterials science, 5 (2017) 432- 443.
6. A. Figuerola, R. Di Corato, L. Manna, T. Pellegrino, From iron oxide nanoparticles towards advanced iron-based inorganic materials designed for biomedical applications, Pharmacological research, 62 (2010) 126-143.
7. Wahajuddin, S. Arora, Superparamagnetic iron oxide nanoparticles: magnetic nanoplatforms as drug carriers, International journal of nanomedicine, 7 (2012) 3445-3471.

8. H. Mok, M. Zhang, Superparamagnetic iron oxide nanoparticle-based delivery systems for biotherapeutics, Expert opinion on drug delivery, 10 (2013) 73-87.
9. J. Szebeni, Complement activation-related pseudoallergy: a new class of drug- induced acute immune toxicity, Toxicology, 216 (2005) 106-121.
10. J. Szebeni, Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals, Mol. Immunol., 61 (2014) 163- 173.
11. J. Szebeni, G. Storm, Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs, Biochemical and biophysical research communications, 468 (2015) 490-497.
12. J. Szebeni, F. Muggia, Y. Barenholz, Case study: Complement activation related hypersensitivity reactions to PEGylated liposomal doxorubicin: Experimental and clinical evidence, mechanisms and approaches to Inhibition, in: M.A. Dobrovolskaia, S.E. McNeil (Eds.) Handbook of Immunological Properties of Engineered Nanomaterials, 2nd Edition, World Scientific Publishing Company, 2015, pp. 331-361.
13. J.A. Jackman, T. Meszaros, T. Fulop, R. Urbanics, J. Szebeni, N.J. Cho, Comparison of complement activation-related pseudoallergy in miniature and domestic pigs: foundation of a validatable immune toxicity model, Nanomedicine, NBM, 12 (2016) 933-943.
14. W. Bayer Healthcare Pharmaceuticals, NJ, Feridex I.V. (ferumoxides injectable solution), https://www.drugs.com/pro/feridex.html, (2017).
15. EMA, EMA Assessment Report, Rienso, http://www.ema.europa.eu/docs/ en_GB/document_library/EPAR_-
16. Assessment_Report_-_Variation/human/002215/WC500184877.pdf, (2015).
17. C. Advanced Magnetics Inc., Oncology, Drug Advisory Committee Biefing Doument, NDA 21-115; 68-71., (2005).
18. V.M. Runge, Safety of approved MR contrast media for intravenous injection, J.Magn Reson.Imaging., 12 (2000) 205-213.
19. V.M. Runge, Safety of magnetic resonance contrast media, Top.Magn Reson.Imaging., 12 (2001) 309-314.
20. G. Wang, J.I. Griffin, S. Inturi, B. Brenneman, N.K. Banda, V.M. Holers, S.M. Moghimi, D. Simberg, In Vitro and In Vivo Differences in Murine Third Complement Component (C3) Opsonization and Macrophage/Leukocyte Responses to Antibody-Functionalized Iron Oxide Nanoworms, Frontiers in immunology, 8 (2017) 151.
21. F. Chen, G. Wang, J.I. Griffin, B. Brenneman, N.K. Banda, V.M. Holers, D.S. Backos, L.

Wu, S.M. Moghimi, D. Simberg, Complement proteins bind to nanoparticle protein corona and undergo dynamic exchange in vivo, Nature nanotechnology, 12 (2017) 387-393.

22. S. Inturi, G. Wang, F. Chen, N.K. Banda, V.M. Holers, L. Wu, S.M. Moghimi, D. Simberg, Modulatory Role of Surface Coating of Superparamagnetic Iron Oxide Nanoworms in Complement Opsonization and Leukocyte Uptake, ACS nano, 9 (2015) 10758-10768.
23. N.K. Banda, G. Mehta, Y. Chao, G. Wang, S. Inturi, L. Fossati-Jimack, M. Botto,
24. L. Wu, S.M. Moghimi, D. Simberg, Mechanisms of complement activation by dextran-coated superparamagnetic iron oxide (SPIO) nanoworms in mouse versus human serum, Particle and fibre toxicology, 11 (2014) 64.
25. G. Wang, S. Inturi, N.J. Serkova, S. Merkulov, K. McCrae, S.E. Russek, N.K. Banda, D. Simberg, High-relaxivity superparamagnetic iron oxide nanoworms with decreased immune recognition and long-circulating properties, ACS nano, 8 (2014) 12437-12449.
26. J.C. Hempel, F. Poppelaars, M. Gaya da Costa, C.F. Franssen, T.P. de Vlaam, M.R. Daha, S.P. Berger, M.A. Seelen, C.A. Gaillard, Distinct in vitro Complement Activation by Various Intravenous Iron Preparations, American journal of nephrology, 45 (2017) 49-59.
27. I.C. Macdougall, K. Vernon, Complement Activation-Related Pseudo-Allergy: A Fresh Look at Hypersensitivity Reactions to Intravenous Iron, American journal of nephrology, 45 (2017) 60-62.
28. B. Romberg, L. Baranyi, R. Bünger, W.-E. hennink, J. Szebeni, G. Storm, Initial observations on complement activation by poly(amino acid)-coated liposomes, in Poly(amino acid)s: next-generation coatings for long-circulating liposomes - Doctoral thesis, Utrecht University, Utrecht, 2007.
29. J.M. van den Hoven, R. Nemes, J.M. Metselaar, B. Nuijen, J.H. Beijnen, G. Storm,
30. J.Szebeni,Complement activation by PEGylated liposomes containing prednisolone, European Journal of Pharmaceutical Sciences, (2013).
31. T. Mészáros, G. Szénási, L. Rosivall, J. Szebeni, L. Dézsi, Paradoxical rise of hemolytic complement in the blood of mice during zymosan- and liposome induced CARPA: a pilot study, Eur. J. Nanomed. , 7 (2015) 257–262.
32. J. Szebeni, P. Bedocs, D. Csukas, L. Rosivall, R. Bunger, R. Urbanics, A porcine model of complement-mediated infusion reactions to drug carrier nanosystems and other medicines, Adv Drug Deliv Rev, 64 (2012) 1706-1716.
33. J. Szebeni, P. Bedocs, R. Urbanics, R. Bunger, L. Rosivall, M. Toth, Y. Barenholz, Prevention of infusion reactions to PEGylated liposomal doxorubicin via tachyphylaxis

induction by placebo vesicles: a porcine model, Journal of controlled release, 160 (2012) 382-387.

34. R. Urbanics, J. Szebeni, Lessons learned from the porcine CARPA model: constant and variable responses to different nanomedicines and administration protocols, Eur J Nanomedicine, 7 (2015) 219-231.
35. P.P. Wibroe, A.C. Anselmo, P.H. Nilsson, A. Sarode, V. Gupta, R. Urbanics, J. Szebeni, A.C. Hunter, S. Mitragotri, T.E. Mollnes, S.M. Moghimi, Bypassing adverse injection reactions to nanoparticles through shape modification and attachment to erythrocytes, Nature nanotechnology, 12 (2017) 589-594.
36. J. Szebeni, F.M. Muggia, C.R. Alving, Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study, J Natl Cancer Inst, 90 (1998) 300-306.
37. J. Szebeni, L. Baranyi, S. Savay, H.U. Lutz, E. Jelezarova, R. Bunger, C.R. Alving, The role of complement activation in hypersensitivity to pegylated liposomal doxorubicin (Doxil®), Journal of liposome research, 10 (2000) 467-481.
38. J. Szebeni, C.R. Alving, S. Savay, Y. Barenholz, A. Priev, D. Danino, Y. Talmon, Formation of complement-activating particles in aqueous solutions of Taxol: Possible role in hypersensitivity reactions, Intern. Immunopharm., 1 (2001) 721- 735.
39. Szebeni, L. Baranyi, S. Savay, J. Milosevits, R. Bunger, P. Laverman, J.M. Metselaar, G. Storm, A. Chanan-Khan, L. Liebes, F.M. Muggia, R. Cohen, Y. Barenholz, C.R. Alving, Role of complement activation in hypersensitivity reactions to doxil and hynic PEG liposomes: experimental and clinical studies, Journal of liposome research, 12 (2002) 165-172.
40. A. Chanan-Khan, J. Szebeni, S. Savay, L. Liebes, N.M. Rafique, C.R. Alving, F.M. Muggia, Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions, Ann Oncol, 14 (2003) 1430-1437.
41. B. Romberg, J.M. Metselaar, L. Baranyi, C.J. Snel, R. Bunger, W.E. Hennink, J. Szebeni, G. Storm, Poly(amino acid)s: Promising enzymatically degradable stealth coatings for liposomes, Int. J. Pharm., 331 (2007) 186-189.
42. J.M. van den Hoven, R. Nemes, J.M. Metselaar, B. Nuijen, J.H. Beijnen, G. Storm, J. Szebeni, Complement activation by PEGylated liposomes containing prednisolone, European journal of pharmaceutical sciences, 49 (2013) 265-271.
43. G.T. Kozma, T. Mészáros, Z. Weiszhár, T. Schneider, A. Rosta, R.-. Urbanics, L. Rosivall,

J.-. Szebeni, Variable association of complement activation by rituximab and paclitaxel in cancer patients in vivo and in their screening serum in vitro with clinical manifestations of hypersensitivity: a pilot study, Eur. J. Nanomedicine, 7 (2015) 289–301.

44. L.W. Starmans, D. Burdinski, N.P. Haex, R.P. Moonen, G.J. Strijkers, K. Nicolay,
45. H. Grull, Iron oxide nanoparticle-micelles (ION-micelles) for sensitive (molecular) magnetic particle imaging and magnetic resonance imaging, PloS one, 8 (2013) e57335.
46. C.W. Jung, P. Jacobs, Physical and chemical properties of superparamagnetic iron oxide MR contrast agents: ferumoxides, ferumoxtran, ferumoxsil, Magnetic resonance imaging, 13 (1995) 661-674.
47. V. Filipe, A. Hawe, W. Jiskoot, Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates, Pharmaceutical research, 27 (2010) 796-810.
48. T. Shen, R. Weissleder, M. Papisov, A. Bogdanov, Jr., T.J. Brady, Monocrystalline iron oxide nanocompounds (MION): physicochemical properties, Magnetic resonance in medicine, 29 (1993) 599-604.
49. M.G. Harisinghani, J. Barentsz, P.F. Hahn, W.M. Deserno, S. Tabatabaei, C.H. van de Kaa, J. de la Rosette, R. Weissleder, Noninvasive detection of clinically occult lymph-node metastases in prostate cancer, The New England journal of medicine, 348 (2003) 2491-2499.
50. FDA, Postmarketing safety review https://www.fda.gov/ohrms/dockets/ac/05/briefing/ 2005-4095B1_02_15-FDA-Tab- 7-10.pdf, (2005).
51. G. Milosevits, Z. Rozsnyay, G.T. Kozma, J. Milosevits, G. Tömöry, H. Robotka, L. Rosivall, J. Szebeni, Flow cytometric analysis of supravesicular structures in doxorubicin-containing pegylated liposomes, Chem. Phys. Lipids, 165 (2012) 482- 487.

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The role of thromboxane A_2 in complement activation-related pseudoallergy

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Abstract

Complement activation-related pseudoallergy (CARPA) is a hypersensitivity reaction occurring upon i.v. administration of numerous liposomal therapeutics, other non-biological complex drugs and biologicals. It has a complex molecular and cellular mechanism that involves the production, actions and interactions of numerous vasoactive mediators in blood, including thromboxane A_2 (TXA_2). This short review focuses on the latter eicosanoid; its role in CARPA, effects underlying some of the symptoms and experimental evidence for its rate limiting role in pulmonary hypertension in pigs. Animal experiments and recent clinical observations suggest that the cyclooxygenase blocker indomethacin, may represent an effective new approach to prevent liposome-induced CARPA, lending clinical relevance to better understand the involvement of TXA2 and other eicosanoids in this adverse immune effect.

Keywords: allergy, immune toxicity, adverse drug reactions, anaphylatoxins; anaphylaxis; animal models; hemodynamic changes; hypersensitivity reactions; pseudoallergy,

Introduction

Numerous state-of-art drugs and imaging agents can cause an acute immune reaction known as complement (C) activation-related pseudoallergy (CARPA): an anaphylactoid or infusion reaction whose rise can be associated with activation of C system (1-9). These can include nanoparticles and nanomaterial such as the liposomal drugs Doxil and AmBisome and micellar solvents containing amphiphilic lipids Cremophor EL. The phenomenon seems to be an intrinsic property of the above agents for the simple reason that they resemble foreign pathogens against which the immune system developed effective defense and thus vehemently responds upon i.v. encounter [10]. The mechanism of CARPA is still poorly understood; it is a complex chain reaction involving numerous cellular and molecular interactions [1].

Figure 1 presents a scheme of the "CARPA cascade", consisting of three rate-limiting processes; [1] activation of the C system; [2] stimulation of blood cells and allergy mediating secretory cells by anaphylatoxins; and [3] triggering the various responses of effector cells (WBC, platelets, endothelial cells and smooth muscle cells) either by the anaphylatoxins, directly, or via multiple allergomedins released by the allergy-mediating secretory cells.

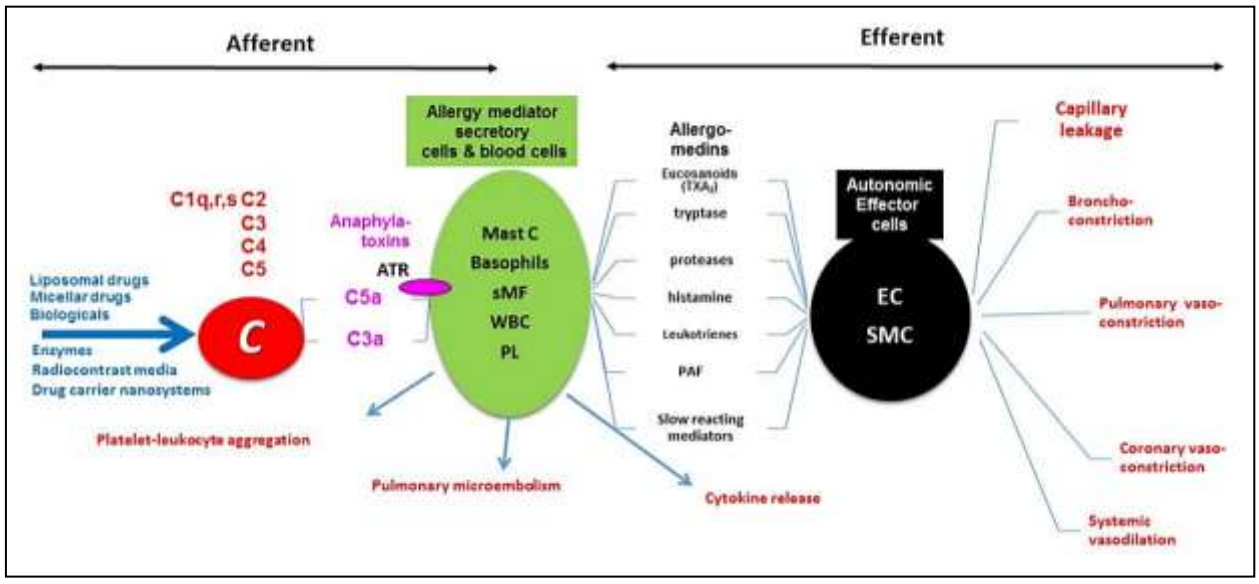

Figure 1. Afferent and efferent arms of CARPA. The hypothetical scheme illustrates the steps and interactions among a great number of cells and mediators involved in CARPA. AR, anaphylatoxin receptors; Mast C, mast cells; sMF, secretory macrophages; WBC, white blood cells. The hypothetical scheme illustrates the steps and interactions among a great number of cells; PL, platelets; EC, endothelial cells; SMC, smooth muscle cells. Modified from ref. [1] with permission.

The first step in the efferent arm is the liberation of numerous vasoactive mediators from a variety of allergy-mediating cells, e.g., mast cells, basophil leukocytes, secretory macrophages, white blood cells and platelets. The mediators, referred to as "allergomedins", include, among others, thromboxane A_2 (TXA_2), tryptase, proteases, histamine, leukotrienes, platelet activating factor (PAF) and slow reacting mediators (such as SRS-A (slow-reacting substance of anaphylaxis) causing anti-histamine resistive prolonged, slow contraction of smooth muscle, or an increase in vascular permeability and mucous secretion by prostaglandins and eosinophil chemotactic factors, etc.) [1]. Among these, this "minireview" focuses only on TXA_2, the eicosanoid that was discovered in the lung perfusate of guinea pigs undergoing anaphylactic shock [11-13]. Anaphylactic shock, ending in cardiac failure (cardiac anaphylaxis) is also the most severe manifestations of rat [14] and porcine CARPA [15]; therefore, it seems logical to connect TXA_2 to CARPA not only in guinea pigs but in other animals and man as well. Beyond these theoretical considerations. there is solid experimental evidence that TXA_2 plays a key, rate limiting role in the pulmonary and cardiac hemodynamic changes in CARPA in pigs and rats. Interestingly, the effects occurring are consistent with recent clinical observations on the efficacy of the cyclooxygenase inhibitor indomethacin in preventing liposome-induced CARPA in cancer patients.

By recapitulating the basic facts about TXA_2 and previous findings on its role in animal models of CARPA, our goal was to draw attention to a possible drug target for pharmaceutical inhibition of this adverse side effect and thus make reactogenic nanomedicines safer.

Basic facts about Thromboxane A2

Thromboxane A_2 **(**TXA_2), a member of the family of lipids known as eicosanoids, is an oxygenated metabolite of arachidonic acid (AA). It is generated from AA by the actions of cyclooxygenase (COX-1 and 2) and thromboxane-synthase (TXS) (Figure 2).

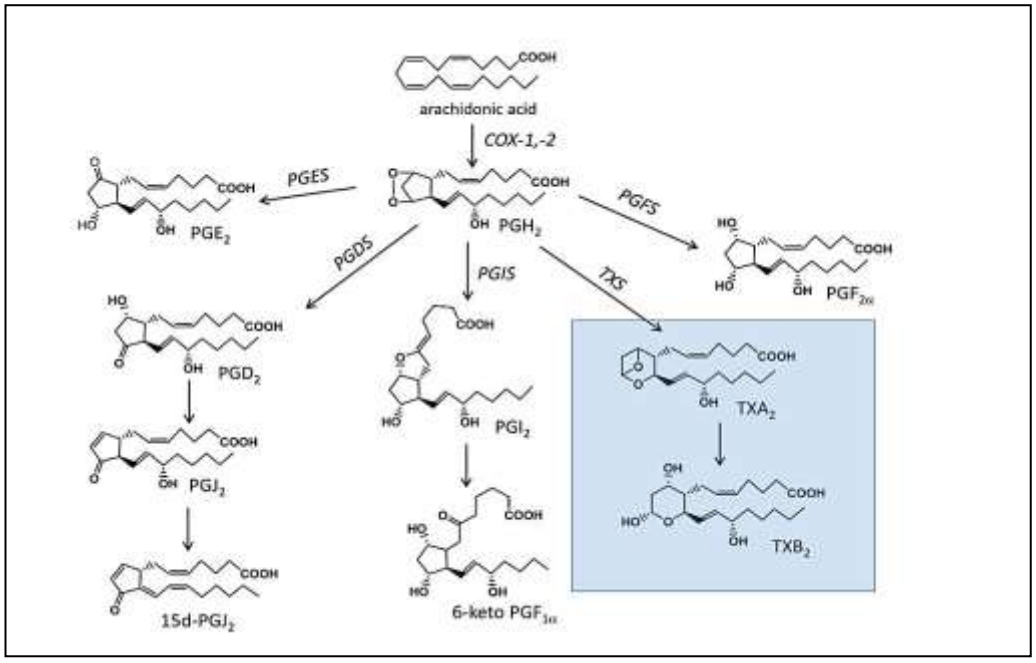

Figure 2. Metabolic pathway of TXA_2 formation and its hydrolysis to TXB_2 (blue shaded area). In addition to these eicosanoids, the scheme shows the biochemical pathways of all main AA metabolites in order to enable the identification of further CARPAgenic prostanoids. Abbreviations: COX, cyclooxygenase; PGES, prostaglandin E synthase; PGDS, prostaglandin D synthase; PGFS, prostaglandin F synthase; PGIS, prostacyclin synthase; TXS, thromboxane synthase. Public image on the internet (http://www.frontiersin.org/files/Articles/77568/fimmu-05-00075-HTML/image_m/fimmu-05-00075-g001.jpg) originating from Ref. [16].

After prostaglandin PGH_2 is synthesized from AA, thromboxane synthase (TXS) helps its rearrangement to TXA_2. Thromboxane synthase is a ferrihemoprotein enzyme anchored to the endoplasmic reticulum of platelets, monocytes and several other cells. In catalyzing the synthesis of thromboxanes, TXS modulates several important physiological processes, such as blood pressure, clotting, and inflammatory responses [17-21].

Thromboxane A_2 is unstable in water and undergoes hydrolysis to TXB_2 (Figure 2) within seconds to minutes. Thus, due to its very short half-life, TXA_2 primarily functions as an autocrine or paracrine mediator in the nearby tissues surrounding its site of production. Thromboxane B_2 is, on the other hand, stable, enabling it to become the standard (surrogate) marker of TXA_2 production. The main source of TXA_2 is activated platelets in blood, but TXA_2 is also produced by many other cells in tissue and blood, e.g., cultured lung fibroblast (WI-38) from guinea pigs [12], macrophages, including pulmonary intravascular macrophages (PIM cells) from rabbits [22], pigs [23] and minipigs [24]. These cells are mentioned because they play a critical role in the pulmonary symptoms of CARPA [23].

As for its biological activity, TXA_2 is a potent vasoconstrictor and pro-coagulant agent [25-33]

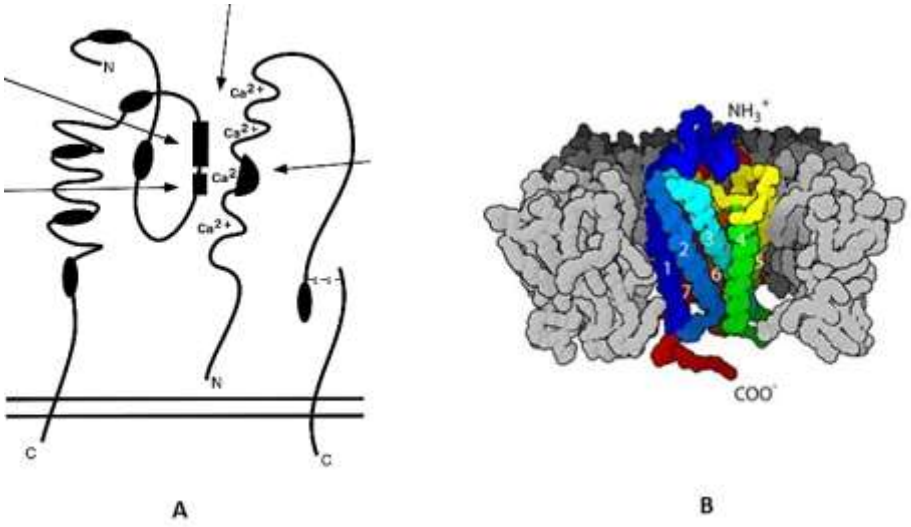

Figure 3. Surface receptors in service of TXA_2 function. A) schematic illustrations of GPIIIa/IIb molecule, which is upregulated by TXA_2 and mediates many of its effects; and B) thromboxane receptor (TP).

Thromboxane A_2 stimulates platelet activation and aggregation, which effect is achieved, at least in part, by upregulation of the GPIIb/IIIa glycoprotein complex on the membrane (Figure 3A). Thromboxane A_2 acts via specific receptors, the thromboxane prostanoid receptor (TP), one among five classes of prostanoid receptors, a G protein–coupled membrane protein containing seven transmembrane α-helical domains (Figure 3B) [34-36]. The TP receptors have 2 isoforms known as TPα and TPβ, which differ in their intracellular C-terminal domains. Functionally, as members of the G-protein-coupled receptor superfamily, engagement of TP receptors by their ligands lead to, among other effects, phospholipase C activation, calcium influx into the cells and activation of protein kinase C. However, the two isoforms differ upon adenylate cyclase coupling: TPα activates while TPβ inhibits the enzyme. Furthermore, because of intrareceptor differences in the C-terminal tail sequence between the two isoforms, TPβ can be internalized as a response to agonist exposure via GRK (G-protein coupled receptor) phosphorylation while whereas TPα cannot. [25-33, 37].

TXB_2 as a laboratory marker of CARPA

The CARPA "syndrome" involves hemodynamic, hematological, skin and laboratory changes (2). Out of this "tetrad" of symptoms, TXB_2 is used as the best "laboratory" biomarker, as in all animal models studied to date its rise was significant and highly reproducible, closely paralleling the hemodynamic and blood cell changes. Figure 4 shows the close parallelism of TXB_2 changes with

the decline of systemic arterial pressure (SAP) and biphasic drop followed by a rise of WBC and platelet counts in rats in response of i.v. injected liposomes (AmBisome) and Zymosan.

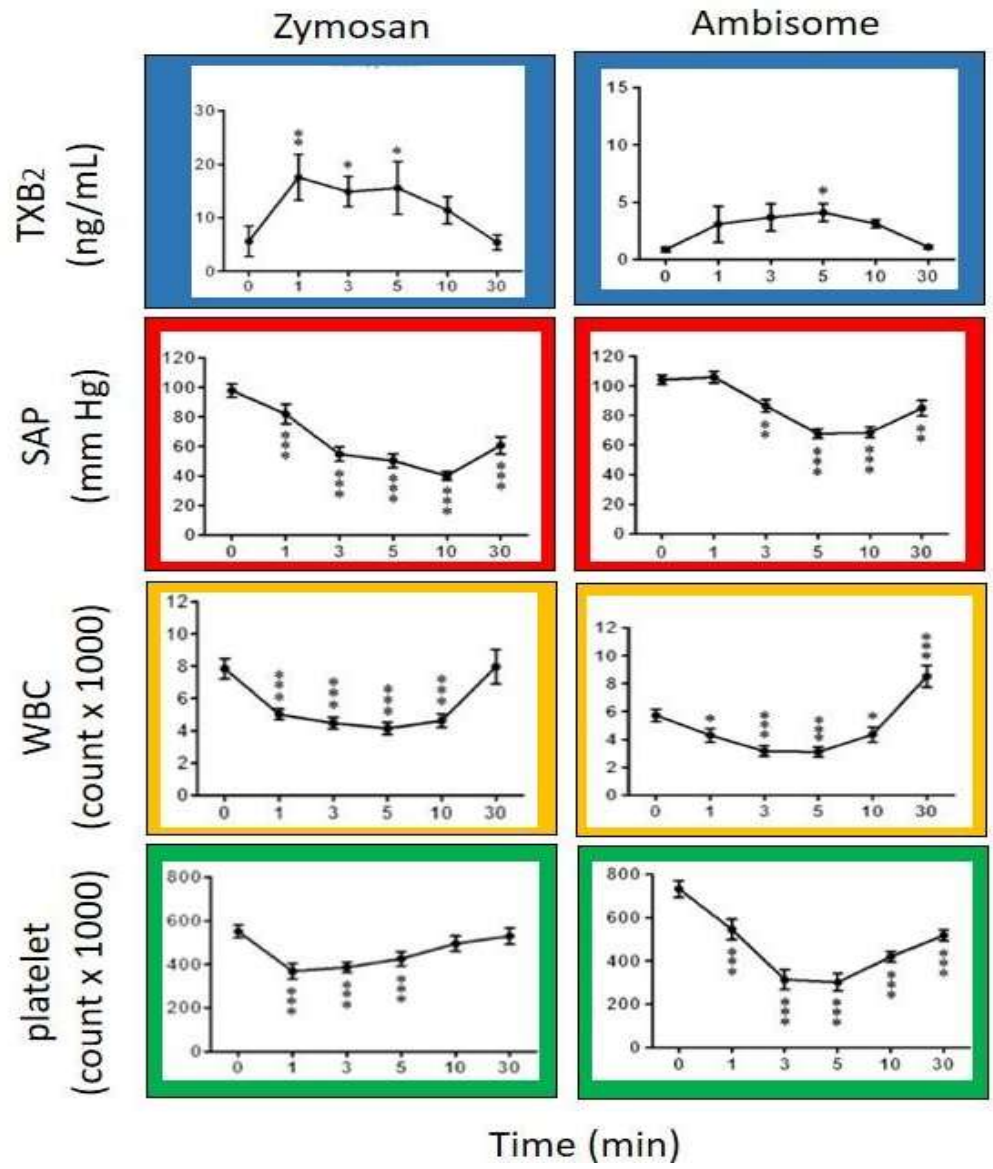

Figure 4. Typical manifestations of CARPA in rats injected with AmBisome (22 mg/kg) and Zymosan i.v., (5 mg/kg). The panels with different background colors are different symptoms, specified on the Y axes. The panels in each column are measurements from the same reaction, averages and SE from 6 rats for each test agent. Figure reproduced from [4] with permission.

Figure 5 shows the changes of TXB_2 and pulmonary arterial pressure (PAP) in domestic and minipigs, injected i.v. with various liposomes or other nanoparticles. Again, these data reveal a remarkable concurrence of kinetics, by which these parameters change on a minute scale during CARPA reactions; they start within 1 minute, peak at 2-3 min and then all parameters return to baseline within 10-15 min.

The role of TXA_2 in CARPA

The above exemplified close correlation between the rise of TXB_2 and other symptoms of CARPA suggests they are causally related. TXA_2, as a vasoconstrictor, has been well known to cause pulmonary hypertension in animals [38, 39], suggesting the likely operation of direct causal relationship between TXA_2 liberation and pulmonary hypertension, at least in pigs. However, additional mechanisms cannot be excluded, such as microcirculatory blockage due to leukocyte and/or platelet adhesion to pulmonary endothelial cells, along with microthrombus formation as a consequence of platelet aggregation and/or leukocyte-platelet aggregate formation (40). These processes are primarily triggered by the anaphylatoxins, C3a and C5a, but, because WBC, platelets, endothelial cells and macrophages also have TXB_2 receptors [34, 35, 37, 41-46] it is likely that TXA_2 also plays an important role in their escalation.

Evidence for direct causal role of TXA_2 in porcine CARPA

Several lines of evidence suggest that pulmonary hypertension in pigs directly depends on TXA_2, rather than C5a production. One of these is the dose-response relationship between PAP and TXB2 before and at different times after injection of various liposomes, data presented in Figure 5A-C. Fig. 5A shows the PAP peak readings as a function of TXB_2 concentration in blood; data compiled from 7 pigs injected with multilamellar dimyristoyl phosphatidilcholine/dimyristoyl phosphatidylglycerol/cholesterol (45/5/50 mole ratios) liposomes (MLV) several times [5]. The highly significant quantitative correlation between these variables, taken together with the remarkable temporal coincidence of their peaks both in case of LMV (Fig. 5B) [5] and Doxil (Fig 5C) [48] provide strong evidence for a direct causal role of TXA_2 in porcine CARPA**.** Furthermore, the finding that this correlation is equally present in non-tachyphylactic (Fig 5B) and tachyphylactic (Fig. 5C) pulmonary responses suggests that the cause of tachyphylaxis is interruption of the "CARPA cascade" [1] at an interaction earlier than TXA_2 formation by macrophages and/or platelets, rather than the loss of TXA_2 sensitivity of CARPA effector cells.

In addition to the significant dose-response relationship discussed above, the other solid evidence for the causal role of TXA_2 in porcine CARPA is the strong inhibitory effect of the COX-inhibitor indomethacin on liposome-induced rise of PAP in pigs [5]. The effect suggests that the liberation of TXA_2 with or without other indomethacin-sensitive pulmonary prostanoids, rather than that of C5a or any other C split product, plays a rate-limiting role in pulmonary hypertension. This is good

news as it implies that CARPA must be sensitive to a large number of drugs inhibiting AA metabolism. Consistent with the pig data [5], preliminary clinical information suggests that indomethacin pretreatment is effective in preventing Doxil-induced CARPA in cancer patients (47).

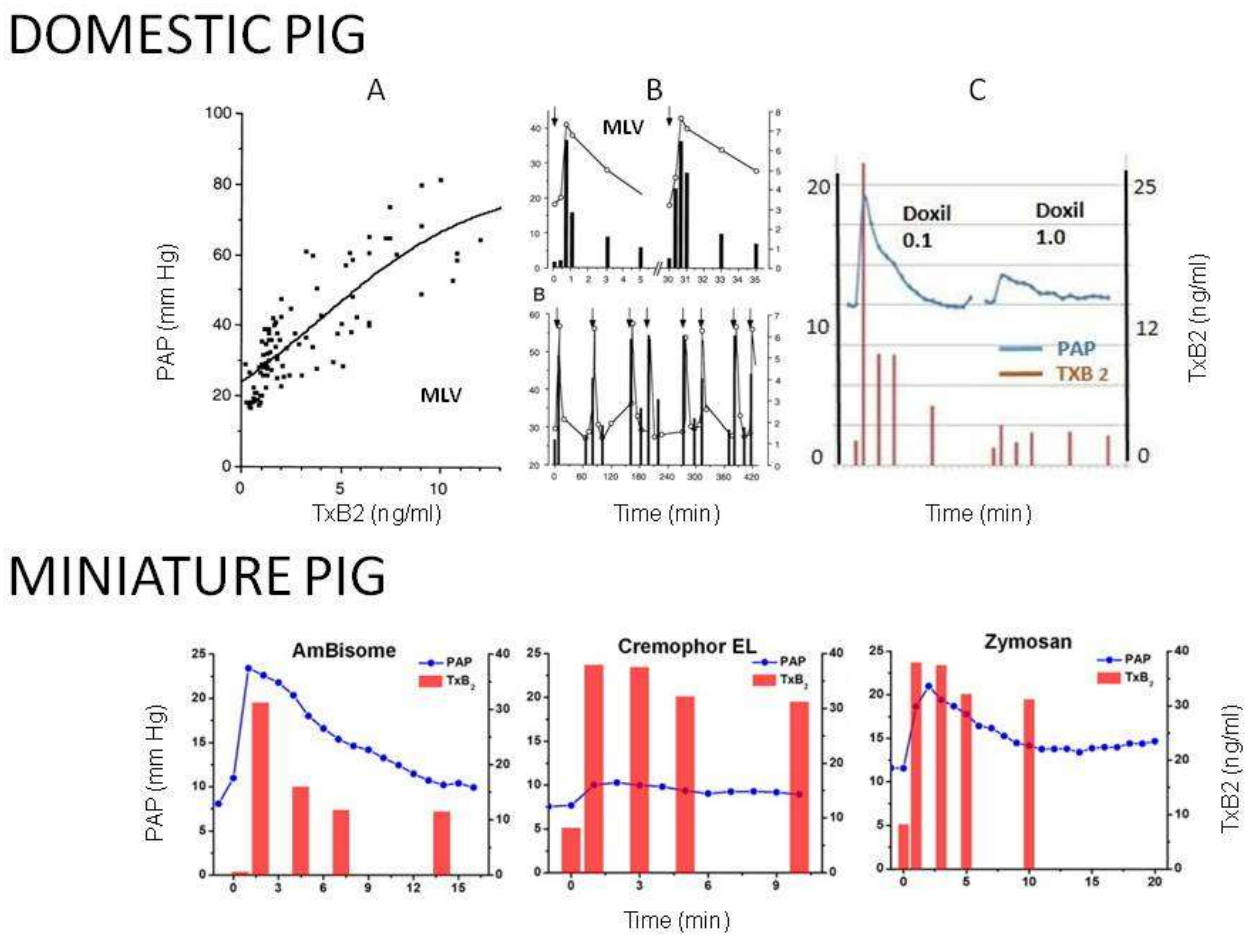

Figure 5. The role of TXA_2 in the pulmonary hypertension of pigs and miniature pigs undergoing liposome-induced CARPA. Upper panels: domestic pigs. A) Strong quantitative correlation between PAP (curve) and plasma TXB_2 levels (dots) in pigs injected with multilamellar liposomes (MLV) consisting of DMPC/DMPG/Chol 50:5:45 mole ratios. B) Kinetic correlation between the rises of PAP (curve) and plasma TXB_2 levels (bars) in a pig sequentially injected with the same dose of MLV. (C) Tachyphylaxis: Doxil sequentially injected in a pig at 0.1 and, then 1 mg/kg, displayed decreasing PAP and TXB_2 response. Lower panels: miniature pigs injected with the specified agents; AmBisome at 1 mg/kg, Cr-EL at 1 mL/kg and Zymosan at 0.5 mg/kg. Plasma TXB_2 was measured by competitive ELISA. Reproduced from [5, 48] with permission.

Indomethacin and its effect on CARPA

Indomethacin, a non-steroidal anti-inflammatory agent (NSAID) with analgesic and antipyretic activities, has been used in the clinics since the mid-sixties [49, 50]. There are different chemical specifications and many spellings for the same agent (Table 1), sold today –according to internet information- by 33 companies under 67 generic names (http://www.medindia.net/drug-price/indomethacin.htm). It is prescribed for a variety of inflammatory diseases and conditions, e.g., against joint pain, fever, swelling and many other common symptoms of inflammation. Indomethacin is a potent inhibitor of prostaglandin synthesis by blocking both cyclooxygenases (COX)-1 and COX-2 [50].

{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl}acetic acid
1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid
Aconip
Indocin
Indometacin
Indometacina
Indometacine
Indometacinum
Indomethacin

Table 1: Chemical names and spellings of indomethacin brand names

Figure 6A reminds of some basic information on indomethacin, while Figure 6B recapitulates the mentioned experiment indicating full inhibition of multilamellar liposome (MLV)-induced CARPA in pigs by 5 mg/kg indomethacin. The latter effect was recently confirmed in many more studies (unpublished observations). Importantly, according to anecdotal evidence, indomethacin has also been used successfully to prevent Doxil- and other liposome-induced hypersensitivity reactions in cancer patients [47].

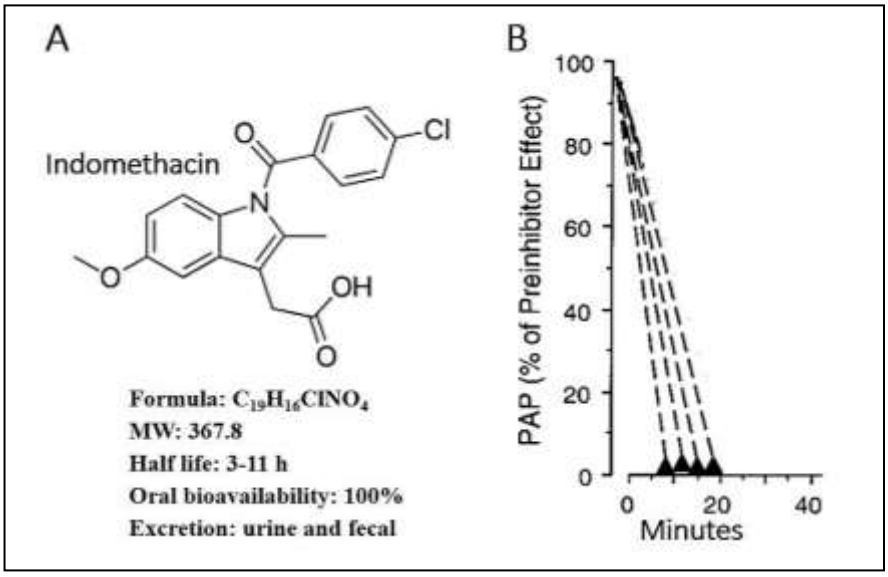

Figure 6. Basic characteristics of indomethacin (A) and its effect on MLV-induced CARPA in pigs (B). In panel B MLV was injected in 4 pigs at time 0 to induce reactions, and the rises of PAP were measured to quantify baseline CARPA. 15 min later, after returning of baseline values, the animals were treated with 5 mg/kg i.v. indomethacin i.v., followed by injection of the same dose of MLV as given at time 0. The maximal rise of PAP at this time was related to the maximal rise of PAP at baseline, and the ratios are given in %.

Outlook

CARPA, an infusion reaction due to C activation, is as safety issue with numerous liposomal therapeutics, non-biological complex drugs and biologicals [3]. Its prevention and improved control are important for the clinical success of these state-of-art medicines. The consistent observation in many studies and different animals over the past decade, that TXA_2 is a key, rate-limiting mediator of hemodynamic changes in CARPA represents a major step towards solving the CARPA problem, since TXA_2 is a prominent product of eicosanoid metabolism whose inhibition at COX is one of the most frequently applied metabolic intervention in human pharmacotherapy. The possibility that by taking relatively cheap and nontoxic NSAIDs to prevent CARPA augurs well for its future clinical testing. The other practical benefit of focusing on thromboxanes, or other AA products generated during CARPA is that they serve as a quantitative biomarker to indicate the severity of reactions. To our experience to date, none of the blood tests correlate so well with the hemodynamic changes in CARPA than TXB_2, providing a valid endpoint for in vivo screening assays for regulatory evaluation of the CARPAgenic effect of drugs under R&D. The need for CARPA testing of liposomes and other nanomedicines, as part of preclinical immune toxicology evaluation, have been expressed by both the US Federal Drug Administration [51] and by the European Medicinal Agency [52].

One of the unsolved questions relating to the role of TXA_2 in CARPA is the source of this mediator in pigs and man. In pigs, it is the pulmonary intravascular macrophages (PIM cells) that are held responsible for the dramatic cardiopulmonary changes during the reactions [23, 53]. PIM cells are known to secrete TXA_2 [23], thus, their primary role in porcine CARPA is consistent with the efficacy of indomethacin. However, PIM cells are not known to be present in the lung of man, except in rare liver diseases [23]. Thus, the uniform efficacy of indomethacin in man, or the possible presence of TXA_2 secreting macrophages in the lung of rare, highly reactive patients, remain questions for future research in this field, as well as the relative contributions of platelet- versus PIM cell-derived TXA_2 to CARPA in pigs and man.

References

1. Szebeni J. Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals. Mol Immunol 2014;61:163-73.
2. Urbanics R, Szebeni J. Lessons learned from the porcine CARPA model: constant and variable responses to different nanomedicines and administration protocols. Eur J Nanomedicine 2015;7;219-231.
3. Szebeni J, Storm G. Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs. Biochemical and biophysical research communications 2015;468:490-7.
4. Dézsi L, Fülöp T, Mészáros T, Szénási G, Urbanics R, Vázsonyi C, et al. Features of Complement Activation-Related Pseudoallergy to Liposomes with Different Surface Charge and PEGylation: Comparison of the Porcine and Rat Responses. J Contr Release 2014:pii: S0168-3659(14)00591-4. doi: 10.1016/j.jconrel.2014.08.009. [Epub ahead of print].
5. Romberg B, Metselaar JM, Baranyi L, Snel CJ, Bunger R, Hennink WE, et al. Poly(amino acid)s: Promising enzymatically degradable stealth coatings for liposomes. Int J Pharm 2007;331:186-9.
6. Szebeni J, Baranyi L, Savay S, Milosevits J, Bunger R, Laverman P, et al. Role of complement activation in hypersensitivity reactions to doxil and hynic PEG liposomes: experimental and clinical studies. Journal of liposome research 2002;12:165-72.
7. van den Hoven JM, Nemes R, Metselaar JM, Nuijen B, Beijnen JH, Storm G, et al. Complement activation by PEGylated liposomes containing prednisolone. European Journal of Pharmaceutical Sciences 2013.
8. Moghimi SM. Complement Propriety and Conspiracy in Nanomedicine: Perspective and a Hypothesis. Nucleic acid therapeutics 2016;26:67-72.
9. Wibroe PP, Ahmadvand D, Oghabian MA, Yaghmur A, Moghimi SM. An integrated assessment of morphology, size, and complement activation of the PEGylated liposomal doxorubicin products Doxil(R), Caelyx(R), DOXOrubicin, and SinaDoxosome. Journal of controlled release : official journal of the Controlled Release Society 2016;221:1-8.
10. Szebeni J, Barenholz Y. Complement activation, immunogenicity, and immune suppression as potential side effects of liposomes. In: Peer D, editor. Handbook of Harnessing Biomaterials in Nanomedicine: Prepara!on,Toxicity, and Applications. Singapure: Pan Standford Publishing Pte.Ltd.; 2012. p. 309-34.

11. Palmer MA, Piper PJ, Vane JR. Release of rabbit aorta contracting substance (RCS) and prostaglandins induced by chemical or mechanical stimulation of guinea-pig lungs. British journal of pharmacology 1973;49:226-42.
12. Piper PJ, Samhoun MN. Stimulation of arachidonic acid metabolism and generation of thromboxane A2 by leukotrienes B4, C4 and D4 in guinea-pig lung in vitro. British journal of pharmacology 1982;77:267-75.
13. Piper PJ, Yaacob HB. Interactions of platelet activating factor, thromboxane A2 and leukotrienes in guinea-pig heart. Progress in clinical and biological research 1989;301:493-8.
14. Baranyi L, Szebeni J, Savay S, Bodo M, Basta M, Bentley TB, et al. Complement-Dependent Shock and Tissue Damage Induced by Intravenous Injection of Cholesterol-Enriched Liposomes in Rats. Journal of Applied Research 2003;3.
15. Szebeni J, Baranyi L, Savay S, Bodo M, Milosevits J, Alving CR, et al. 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.
16. Nicolaou A, Mauro C, Urquhart P, Marelli-Berg F. Polyunsaturated Fatty Acid-derived lipid mediators and T cell function. Frontiers in immunology 2014;5:75.
17. Sakariassen KS, Alberts P, Fontana P, Mann J, Bounameaux H, Sorensen AS. Effect of pharmaceutical interventions targeting thromboxane receptors and thromboxane synthase in cardiovascular and renal diseases. Future cardiology 2009;5:479-93.
18. Wang LH, Kulmacz RJ. Thromboxane synthase: structure and function of protein and gene. Prostaglandins & other lipid mediators 2002;68-69:409-22.
19. Vermylen J, Deckmyn H. Thromboxane synthase inhibitors and receptor antagonists. Cardiovascular drugs and therapy 1992;6:29-33.
20. Fiddler GI, Lumley P. Preliminary clinical studies with thromboxane synthase inhibitors and thromboxane receptor blockers. A review. Circulation 1990;81:I69-78; discussion I9-80.
21. FitzGerald GA, Reilly IA, Pedersen AK. The biochemical pharmacology of thromboxane synthase inhibition in man. Circulation 1985;72:1194-201.
22. Duke-Novakovski T, Singh-Suri S, Kajikawa O, Caldwell S, Charavaryamath C, Singh B. Immuno-phenotypic and functional characterization of rabbit pulmonary intravascular macrophages. Cell Tissue Res 2013;351:149-60.

23. Csukas D, Urbanics R, Weber G, Rosivall L, Szebeni J. Pulmonary intravascular macrophages: prime suspects as cellular mediators of porcine CARPA. Eur J Nanomed 2015;7:27-36.
24. Bertram TA, Overby LH, Danilowicz R, Eling TE, Brody AR. Pulmonary intravascular macrophages produce prostaglandins and leukotrienes in vitro. Chest 1988;93:82S-4S.
25. Radomski M. The biological role of thromboxane A2 in the process of hemostasis and thrombosis; pharmacology and perspectives of therapeutical use of thromboxane synthetase inhibitors and receptor PGH2/TXA2 antagonists. Acta physiologica Polonica 1985;36:153-64.
26. Bauer J, Ripperger A, Frantz S, Ergun S, Schwedhelm E, Benndorf RA. Pathophysiology of isoprostanes in the cardiovascular system: implications of isoprostane-mediated thromboxane A2 receptor activation. British journal of pharmacology 2014;171:3115-31.
27. Ting HJ, Murad JP, Espinosa EV, Khasawneh FT. Thromboxane A2 receptor: biology and function of a peculiar receptor that remains resistant for therapeutic targeting. Journal of cardiovascular pharmacology and therapeutics 2012;17:248-59.
28. Kontogiorgis C, Hadjipavlou-Litina D. Thromboxane synthase inhibitors and thromboxane A2 receptor antagonists: a quantitative structure activity relationships (QSARs) analysis. Current medicinal chemistry 2010;17:3162-214.
29. Feletou M, Vanhoutte PM, Verbeuren TJ. The thromboxane/endoperoxide receptor (TP): the common villain. Journal of cardiovascular pharmacology 2010;55:317-32.
30. Armstrong RA, Wilson NH. Aspects of the thromboxane receptor system. General pharmacology 1995;26:463-72.
31. Hall SE. Thromboxane A2 receptor antagonists. Medicinal research reviews 1991;11:503-79.
32. Patscheke H. Thromboxane A2/prostaglandin H2 receptor antagonists. A new therapeutic principle. Stroke 1990;21:IV139-42.
33. Patrono C. Thromboxane synthesis inhibitors and receptor antagonists. Thrombosis research Supplement 1990;11:15-23.
34. Quinton TM, Murugappan S, Kim S, Jin J, Kunapuli SP. Different G protein-coupled signaling pathways are involved in alpha granule release from human platelets. Journal of thrombosis and haemostasis : JTH 2004;2:978-84.
35. Murugappan S, Shankar H, Kunapuli SP. Platelet receptors for adenine nucleotides and thromboxane A2. Seminars in thrombosis and hemostasis 2004;30:411-8.

36. Shankar H, Murugappan S, Kim S, Jin J, Ding Z, Wickman K, et al. Role of G protein-gated inwardly rectifying potassium channels in P2Y12 receptor-mediated platelet functional responses. Blood 2004;104:1335-43.
37. Davi G, Santilli F, Vazzana N. Thromboxane receptors antagonists and/or synthase inhibitors. Handbook of experimental pharmacology 2012:261-86.
38. Al-Naamani N, Sagliani KD, Dolnikowski GG, Warburton RR, Toksoz D, Kayyali U, et al. Plasma 12- and 15-hydroxyeicosanoids are predictors of survival in pulmonary arterial hypertension. Pulmonary circulation 2016;6:224-33.
39. Al-Naamani N, Palevsky HI, Lederer DJ, Horn EM, Mathai SC, Roberts KE, et al. Prognostic Significance of Biomarkers in Pulmonary Arterial Hypertension. Annals of the American Thoracic Society 2016;13:25-30.
40. Patkó Z, Szebeni J. Blood cell changes in complement activationrelated pseudoallergy. Eur J Nanomed 2015;7:233–44.
41. Geng L, Wu J, So SP, Huang G, Ruan KH. Structural and functional characterization of the first intracellular loop of human thromboxane A2 receptor. Archives of biochemistry and biophysics 2004;423:253-65.
42. Huang JS, Ramamurthy SK, Lin X, Le Breton GC. Cell signalling through thromboxane A2 receptors. Cellular signalling 2004;16:521-33.
43. Halushka PV, Allan CJ, Davis-Bruno KL. Thromboxane A2 receptors. Journal of lipid mediators and cell signalling 1995;12:361-78.
44. Halushka PV, Matsuda K, Masuda A, Ruff A, Morinelli TA, Mathur RS. Testosterone regulation of platelet and vascular thromboxane A2 receptors. Agents and actions Supplements 1995;45:19-26.
45. Kinsella BT, O'Mahony D, Lawson JA, Pratico D, Fitzgerald GA. Cellular activation of thromboxane receptors. Annals of the New York Academy of Sciences 1994;714:270-8.
46. Kinsella BT, O'Mahony DJ, FitzGerald GA. Phosphorylation and regulated expression of the human thromboxane A2 receptor. The Journal of biological chemistry 1994;269:29914-9.
47. Szebeni J, Muggia F, Barenholz Y. Case study: Complement activation related hypersensitivity reactions to PEGylated liposomal doxorubicin: Experimental and clinical evidence, mechanisms and approaches to Inhibition. In: Dobrovolskaia MA, McNeil SE, editors. Handbook of Immunological Properties of Engineered Nanomaterials, 2nd Edition: World Scientific Publishing Company; 2015. p. 331-61.

48. Jackman JA, Meszaros T, Fulop T, Urbanics R, Szebeni J, Cho NJ. Comparison of complement activation-related pseudoallergy in miniature and domestic pigs: foundation of a validatable immune toxicity model. Nanomedicine : nanotechnology, biology, and medicine 2016;12:933-43.
49. Hart FD, Boardman PL. Indomethacin: A New Non-Steroid Anti-Inflammatory Agent. British medical journal 1963;2:965-70.
50. Ferreira SH, Moncada S, Vane JR. Indomethacin and aspirin abolish prostaglandin release from the spleen. Nature: New biology 1971;231:237-9.
51. Hastings K, L,. Implications of the new FDA/CDER immunotoxicology guidance for drugs. Int Immunopharmacol 2002;11:1613-8.
52. Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product2013.
53. Szebeni J, Bedocs P, Csukas D, Rosivall L, Bunger R, Urbanics R. A porcine model of complement-mediated infusion reactions to drug carrier nanosystems and other medicines. Advanced drug delivery reviews 2012;64:1706-16.

5

The possible role of factor H in complement activation-related pseudoallergy (CARPA): a failed attempt to correlate blood levels of FH with liposome-induced hypersensitivity reactions in patients with autoimmune disease

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Abstract

Factor H (FH) is a natural inhibitor of the alternative pathway (AP) of complement (C) activation, an abundant protein in blood whose reduced level has been associated with proneness for increased C activation. There are also 5 FH-related proteins (FHR), which have different impacts on C function. After brief outlines of the C system and its activation via the AP, this review focuses on FH and FHR, collecting data from the literature that suggest that reduced levels or function of FH is associated with C activation-related hyper- sensitivity reactions (HSRs), called C activation related pseudoallergy (CARPA). Based on such observations we initiated the measurement of FH in the blood of patients with inflammatory bowel disease (IBD) and rheumatoid arthritis (RA), and examined the correlation between FH levels and HSRs following i.v. administration of PEGylated liposomal prednisolone phosphate (PLPP). ELISA assay of FH was conducted on plasma samples before treatment, immediately after treatment and at follow-up visits up to 7 weeks, and an attempt was made to correlate the FH levels obtained with the presence or absence of HSR that occurred in five of twenty patients. However, the initial data presented here on three reactive and three non-reactive patients showed FH levels 600 ug/mL, while the normal range of FH is 2–300 ug/mL. This unexpected outcome of the test led us to realize that the ELISA we used was based on antibodies raised against the short consensus repeats (SCR) in FH, which are also present in FHR. Thus, the

kit cannot distinguish these proteins and we most likely measured the combined levels of FH and FHR. These initial data highlighted an unforeseen technical problem in assessing FH function when using a FH ELISA that cross reacts with FHR, information that helps in further studies exploring the role of FH in CARPA.

Keywords: chronic inflammatory disease; complement; FHR; hypersensitivity reactions; liposomes.

Introduction

A PRIMER IN COMPLEMENT AND FACTOR H

The complement (C) system

One of the major tasks of the C system is to mark and dispose of potentially dangerous particles such as pathogenic microbes and altered host cells [1]. This is achieved by the recognition of and targeted activation on foreign surfaces and modified host targets, such as apoptotic cells. The classical and lectin C pathways are activated upon recognition of certain molecular patterns associated with microbes or altered self, whereas the alternative pathway is activated constantly at a low rate and in an indiscriminative manner. Importantly, complement regulators protect the host from bystander damage.

Artificial surfaces such as stents, cannulae, nanoparticles, liposomes etc. represent foreign surfaces that may cause activation of the complement system. Complement activation can occur via the three above-mentioned path- ways and may proceed, if not attenuated by regulators, to the initiation of the terminal pathway. The terminal pathway is activated upon the cleavage of C5, which generates the inflammatory mediator C5a, and C5b, which by binding additional components can result in the formation of terminal complement complexes (C5b-9 or TCC). C5b-9 when inserted in the target cell membrane forms pores (termed membrane attack complex; MAC) that result in lysis.

The alternative pathway of C activation

The internal thioester bond in C3 can undergo spontaneous hydrolysis, resulting in C3(H2O), which is able to form the C3 converting enzyme C3(H2O)Bb. This enzyme cleaves C3 molecules into

C3a and C3b. C3a is an anaphylatoxin and C3b can bind covalently via its thioester group to nearby hydroxyl or amino groups thus C3b can deposit to target surfaces. In addition, C3b can form fluid phase or surface alternative pathway C3 convertase enzyme (C3bBb). Because each of the three complement path- ways leads to the cleavage of C3 into C3b, the alternative pathway can amplify the cascade reaction started by any pathway (called the "amplification loop"). Thus, even if initial activation is due to the classical or lectin pathway, alternative pathway activity may be responsible for the majority of observed total complement activation [1, 2].

Because of this amplification function of the alternative pathway, its proper regulation in the host is particularly important in order to maintain cell and tissue integrity [2]. Indeed, a number of complement regulatory proteins, both fluid-phase inhibitors and cell membrane bound proteins, act at the C3 level [3].

This also implies that therapeutic alternative pathway inhibitors, particularly those acting at C3/C3b, such as FH, can effectively down-regulate C3 fragment deposition and terminal pathway activation.

FH STRUCTURE AND FUNCTION

FH is the main inhibitor of the alternative complement pathway (reviewed in [4, 5]) (Figure 1A). It is a 155-kDa plasma glycoprotein with a serum concentration of 250 μg/mL. It is a cofactor for factor I in the enzymatic degradation of C3b and also inhibits the C3bBb convertase by preventing its assembly and, when C3bBb already formed, accelerating its decay. Factor H is composed of 20 short consensus repeat (SCR) domains (also termed complement control protein domains, CCPs) (Figure 1B).

The cofactor and decay accelerating activities reside in the N-terminal SCRs 1-4, which also represent one of the two main C3b binding sites within the molecule. The C-terminal SCRs 19-20 contain the second main C3b binding site and this site interacts with the TED domain (residing in the C3d fragment) of the C3b molecule. These domains also harbor a major binding site for cell surface polyanionic host markers, such as sialic acid and glycosaminoglycans. This allows factor H to bind to C3b/C3d deposited on host cell surfaces and thus to discriminate between self and

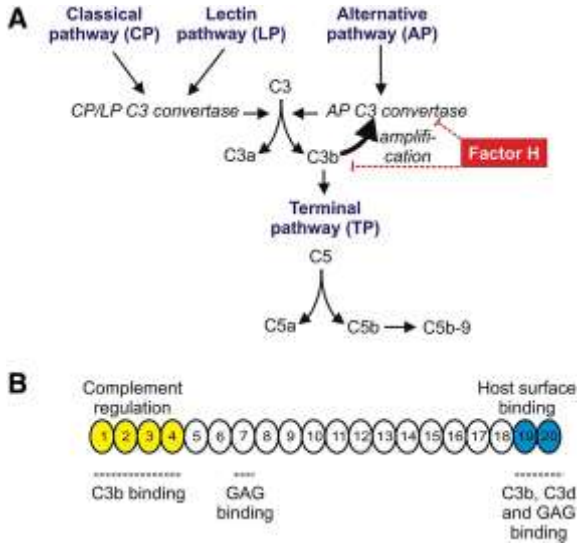

Figure 1. Complement activation and the role of factor H.

(A) Complement is activated via three major pathways, the classical, the lectin and the alternative pathway. Activation generates C3 con- verting enzymes that cleave C3 into C3a and C3b. C3b feeds into the alternative pathway, allowing an amplification loop of complement activation. C3b also binds to the C3 convertases which then are able to cleave C5 into inflammatory C5a and C5b, which by binding additional components can form lytic C5b-9. Factor H inhibits the AP by facilitating the cleavage of C3b by factor I and the inactivation of the AP C3 convertase. (B) Schematic structure of FH. FH is composed of 20 SCR domains. SCRs 1-4 mediate the complement regulatory functions of FH and SCRs 19-20 allow binding of FH to host cell and tissue surfaces. C3b binding sites are located in the same domains; in addition, glycosaminoglycan (GAG) binding sites are found in SCR7 and SCRs 19-20.

non-self [6]. Thus, Factor H acts in the fluid phase (i.e., in plasma and other body fluids) and also inhibits complement activation on host surfaces, such as cells and basement membranes, where it can bind via its glyosaminoglycan/sialic acid binding site. In contrast, microbes normally do not express host-like polyanionic molecules and factor H cannot bind to them, allowing C3b deposition unchecked and progression of the activation cascade [4].

FH is the prototypical member of a family of related proteins, which include FH-like protein 1 (FHL-1), a 42-kDa serum glycoprotein derived via alternative splicing from the FH gene, and five FH-related proteins (FHR-1 to FHR-5) that derive from separate genes adjacent to the FH gene (Figure 2) (reviewed in [7]), FHL-1 includes the SCRs 1-7 of FH plus a four amino acid-long unique C-terminal end and possesses FH-like cofactor and convertase decay accelerating activities. In contrast to this, the five FHR proteins lack domains related to the SCRs 1-4 of FH and lack such activities. The FHRs have 4-9 SCR domains that exhibit various degrees of amino acid sequence

identity to certain FH domains, ranging from distant homology (e.g., 36– 42% of SCRs 1-2 of FHR-1, FHR2 and FHR-5 to SCRs 6-7 of FH) to high similarity (e.g., 95–100% in the three C-terminal SCRs of FHR-1 to FH domains SCRs 18-20).

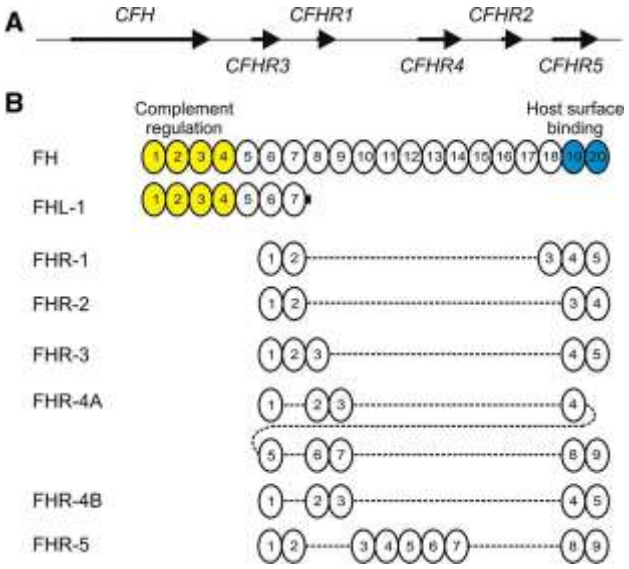

Figure 2. FH-like proteins.

(A) In humans, six genes on the long arm of chromosome 1 encode the members of the FH protein family. The CFH gene gives rise to FH and FHL-1 and five CFHR genes encode the FHR proteins. (B) The domain composition of the FH, FHL-1 and FHR proteins. FH is composed of 20 SCR (or CCP) domains. FHL-1 is identical to the N-terminal SCRs 1-7 of FH plus includes 4 aminoacids at its C terminus. The individual FHR proteins include four to nine SCRs, which are shown in vertical alignment to the homologous domains of FH. The similarity of homologous domains varies from 32 to 100% (not shown in the figure).

Recent data suggest that the FHR proteins can antagonize FH function by competing with FH for ligands such as C3b, thus these proteins seem to enhance complement activation through this mechanism termed de-regulation [8–10]. Thus, the relative amounts of FH and FHRs as well as their affinities to common ligands and surfaces will determine the degree of AP C inhibition by FH locally. A further important consequence of the existence of these closely resembling proteins is that most antibodies, both monoclonal and polyclonal, raised against FH cross-react with one or more of the FHR proteins and FHL-1. Thus, simple immunostaining or ELISA detecting “FH” may be misleading if there is no precise information available on the cross- reactivity of the applied Abs.

FH DEFICIENCY IN DISEASES

There are a number of pathological cases arising from FH dysfunction due to its central role in complement regulation [4, 11]. Overactive FH leads to reduced complement activity against pathogenic agents, thus increasing the susceptibility to microbial infections. However, underactive FH can increase unwanted C activity against healthy host cells leading to complement-mediated disease. These pathologies usually are the results of mutations in the protein or polymorphism. As an example, patients with homozygous FH deficiency leading to underactive FH can suffer from recurrent bacterial infections like Neisseria sp., furthermore vasculitis and glomerulonephritis. In studies of patients with hypocomplementemic glomerulonephritis heterozygous deficiency with mutations of conserved cysteine residues in SCR 9 and 16 were described. These mutations disrupt intrachain disulfide bridges, thereby perturbing the higher order structure of FH. This leads in turn to a profound selective block in secretion of the FH [12]. On the other hand, recent studies showed hyperactivation of the alternative C pathway in neuropsychiatric disorders like schizophrenia: single nucleotide polymorphisms (SNPs) of gene encoding FH was found, CFH rs424535 (2783-526T□A) SNP was positively associated with schizophrenia. Moreover, SNPs rs800292(184G□A) was positively associated with stroke in ischemic stroke studies [13].

FH DEFICIENCY CORRELATING WITH RADIOCONTRAST REACTIONS

A thorough survey of the literature on the relationship between FH levels and HSRs in patients led to the group of Phillip Lieberman et al., who published two papers focusing on HSRs to radiocontrast agents [14, 15]. Radiocontrast agents are a type of medical contrast medium used to improve the visibility of internal bodily structures in X-ray based imaging techniques such as computed tomography (CT) and radiography. They analyzed the adverse reactions to metrizamide, iohexol, ioversol, iopamadol and concluded that next to penicillin these contrast materials are the most significant causes of anaphylactoid reactions worldwide. According to the quoted statistics, the above iodinated radiocontrast materials caused HSRs in approximately 5–9% of patients treated.

Importantly, among the risk factors that showed significant association with radiocontrast reactions in the studies of Lieberman et al., reduced FH levels or FH dysfunction were listed. Radiocontrast reactions, on the other hand, are known to be caused, at least in part, by C activation [16, 17]. The two facts taken together suggest that reduced FH levels or FH dysfunction might sensitize people for not only radiocontrast agent-induced, but all drug-induced CARPA because of reduced natural suppression of C activation via the AP. Consequently, reduced FH levels can be used as a laboratory predictor of i.v. drug- induced CARPA.

FH – AS PREDICTOR OF LIPOSOME- INDUCED HSRS

Based on the above theory on the potential use of FH as a biomarker for drug-induced CARPA, we recently carried out a study wherein the protein profile in the plasma of a normal human subject was analyzed, who showed prone- ness for C activation by liposomal doxorubicin (Caelyx) in vitro (referred to as Caelyx-sensitive plasma, CSP) [18]. The aim was to find one or more specific changes that could be considered as a biomarker for increased susceptibility for C activation. The proteome profiling was done with a library of human plasma proteome specific mAbs on chips (PlasmaScan-380TM) that have been printed on a micro- scope slide-sized glass plate (six/plates), each containing 380 different mAbs raised against non-redundant (with respect to epitopes). The analysis revealed 8 proteins that were differentially represented in CSP in comparison with Caelyx-insensitive control plasma (Table 1).

Among the significant changes we found that the level of FH decreased 1.9-fold in CSP, while the level of FHR was increased 3.4-fold (Table 1) [18]. The decrease in FH was consistent with proneness for increased C activation, while the increase of FHR was difficult to interpret.

In another recent study Kuznetsova et al. showed the presence of FH, along with C3b, among the proteins bound in plasma to liposomes loaded with diglycerade conjugates of melphalan and methotrexate (Mlph-DOG and MTX-DOG). Interestingly, neither fragment C3 nor FH was detected in the protein “corona” in the absence or decreased MTX-DOG content, suggesting that the drug has significant impact on C protein deposition on liposomes. It was concluded that the liposome composition defines the surface properties, which in turn determines the set of plasma proteins bound and thus causes inertness or reactivity of liposomes in circulation [19].

The above information on an inverse correlation between FH levels and risk of HSR to radiocontrast reactions, the in vitro preliminary data on FH levels in a hypersensitive serum and the information on liposome content influencing FH binding to liposomes led us to analyze samples from the clinical study described below to further our understanding of the role of FH in HSRs to i.v. drugs, including liposomes.

Table 1. Protein representation changes in Caelyx sensitive relative to Caelyx insensitive plasmas.

Protein	Direction of change	Fold change: Caelyx vs. Caelyx insensitive plasmas		
FH	Down	**–1.89±0.03**	**–2.25±0.68**	–1.08±0.05
FH and fHRP	Up	**3.42±0.41**	1.88±1.35	1.03±0.71
Serum amyloid P	Up	**4.40±1.33**	1.78±1.59	**7.53±5.97**
Fibronectin	Up	1.37±0.48	**3.23±0.75**	1.35±0.61
Apolipoprotein B100	Up	1.33±0.12	1.35±0.15	**1.66±0.38**
Alpha-2-HS-glycoprotein	Up	1.66±0.45	2.21±1.98	**2.54±1.62**
C4A	Up	**1.90±0.45**	1.47±1.05	0.86±0.44
Protrombin	Up	1.49±1.09	**2.37±1.10**	1.01±0.89

Values in the table show the averaged global normalized intensity ratio of the different plasma samples (mean±SD). Bolded entries are ratios when the corresponding residuals were higher than 2 x of SD of the calculated residual mean. The table shows the monoclonal antibody array-bound labeled proteins, whose raw and globally normalized pixel intensities were significantly lower (down) or higher ("up") than those in (3 different) Caelyx insensitive plasma samples. These proteins repeatedly showed statistically significant deviation from the microarray's inherent variations in at least two out of 3 similar hybridizations. Their identities were determined via immunoprecipitation using dynabeads, followed by MS and Western blot analyses, as described. Table reproduced from [18] with permission.

Preliminary results of a clinical study testing the correlation between FH levels in the blood of PEGylated liposomal prednisolone phosphate-treated patients and rise of HSRs

Patients and Samples

Blood samples were collected from patients suffering from autoimmune diseases (inflammatory bowel disease and rheumatoid arthritis), who participated in two clinical trials wherein they were treated with PEGylated-liposomal prednisolone phosphate (PLPP) administered in infusion. All

participants provided written informed consent. The clinical trials were approved by the local institutional review board and conducted according to the principles of the International Conference on Harmonisation Good Clinical Practice guidelines (Clinicaltrials.gov registration NCT01039103, NCT01647685). In total 22 patients were treated with two injections of PLPP (150 mg) with a two-week interval. Plasma samples were taken before treatment, immediately after treatment and at times of their follow-up visits, initially weekly, later two-weekly. Samples were stored at –80C until shipping to Hungary on dry ice for the FH assays, in the framework of collaboration. Further details and ultimate evaluation of the clinical study will be described elsewhere.

FH ELISA

The human FH ELISA is a standard immune assay using primary monoclonal and secondary (peroxidase bound) antibodies which recognize the SCR on the FH molecule (Figure 1). The HK342 Human Complement FH ELISA kits were obtained from TECOmedical (HK342 Human Complement FH ELISA kit, Hycult Biotech, Uden, The Netherlands). The assay was performed by following the manufacturer's instructions.

Clinical observations

Hypersensitivity reactions were seen in 5 out of 22 patients in these studies. Additionally, two other subjects experienced mild symptoms consistent with an infusion reaction which were not reported as such. Symptoms by which an infusion reaction could be identified were erythema, shortness of breath, itching, flushing and shivers. The infusion reactions were mostly reported as mild and resolved without sequelae after temporarily halting the infusion. In one case a fever was reported (39°C).

Plasma FH levels

After appropriate dilutions, all OD values in the test samples were within the specified effective dynamic range of the assay (5–120 ng/mL) (Figure 3), thus, the ELISA proved to work as expected. Figure 4A and B show the FH levels in the blood of patients displaying or not displaying HSRs, respectively.

The values did not show any trend in initial levels or subsequent changes that would reflect a role in the rise of HSRs. However, it is important to point out that FH levels were in the 600–3000 ug/mL range. According to literature, while previous studies reported plasma FH concentrations ranging 265–684 ug/mL [20], recent studies using monoclonal antibodies reported mean FH concentrations of 233 ug/mL and 269 ug/mL [21]. On the contrary, the FH ELISA kit used in the current study measured 400–800 ug/mL normal range according to its manual. So even if we accept

the higher normal range, in the group of patients with HSR, patient 23 had higher baseline (pre-infusion sample) value than the other two shown, and also the samples taken from this patient were abnormally

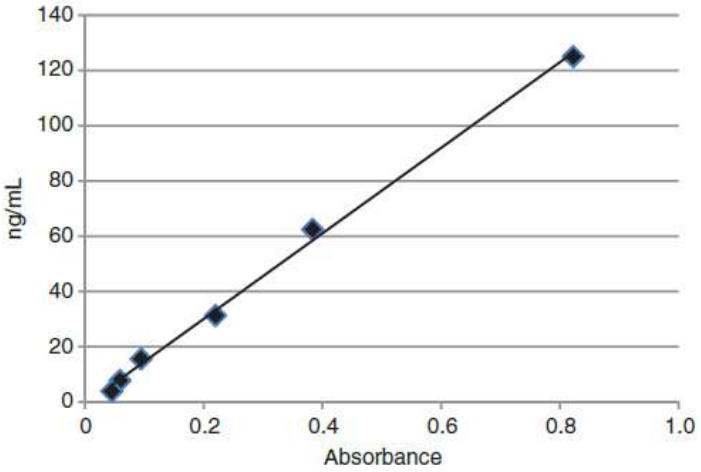

Figure 3: Standard curve of the Hycult FH ELISA kit.

higher than the other patients'. At the patient group with no HSR, patient 09 had a high baseline (pre-infusion), and patient 9 and 11 had abnormally high FH values (See Figure 4A and B). These observations can be most easily rationalized by the fact that the ELISA was not measuring only FH but also FHR, which also carry the SCR antigen for which the ELISA was developed. Alternatively, the high FH levels could be attributed to the autoimmune disease of the patients, although the major rise we observed would be unexpected on the basis that autoimmune diseases are often associated with FH deficiency (see earlier comments on FH diseases).

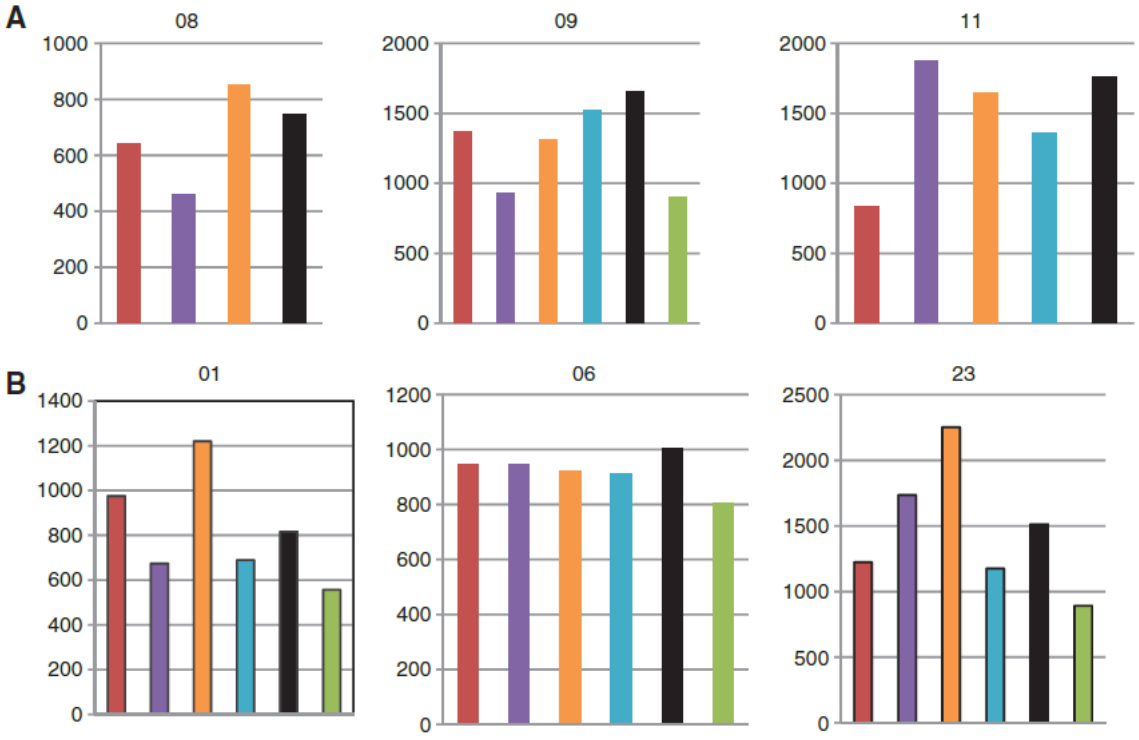

Figure 4. FH levels in patients who did not display HSR during treatment with PLPP (A) and in those who got HSR (B). Samples were taken pre and post treatment, then at 4, 5, 6 and 7 weeks later. Factor H levels were measured in the samples as described in the methods. Color code: Red: Pre Inj., Purple: Post inj., Orange: week 4, blue: week 5, Black: week 6, Green: Week 7. Y values, representing FH concentrations, are given in ug/mL. The numbers above the charts are patient numbers.

DISCUSSION AND OUTLOOK

Complement activation induced by CARPA-genic materials, such as liposomes and radiocontrast media, may be inhibited by engineered or natural C inhibitors. Therefore, it is plausible that levels of such natural inhibitors like FH in plasma of patients with HSRs can influence proneness to and severity of HSRs. Indeed, previous studies suggested that FH levels may be correlated with severity of HSRs [14, 15]. However, determination of FH levels is complicated by the presence of at least six other related proteins (FHL-1 and FHRs) in plasma/serum samples [4]. Increased FHR levels could also result in less regulation by FH due to their antagonistic effect on FH function via competition with FH for certain ligands and surfaces. This may explain the association of increased FHR protein level in Caelyx sensitive plasma compared with Caelyx non-sensitive plasma [18].

The FH measurements presented here did not show any possible correlation between FH level changes and HSR occurrence, most likely because the FH ELISA kit measures not only FH but

also FHR, which also carry the SCR antigen for which the ELISA was developed. For over- coming of this technical barrier, a truly FH specific ELISA would be needed that excludes any FH-related proteins in the measurements.

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References

1. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010;11:785–97.
2. Thurman JM, Holers VM. The central role of the alterna- tive complement pathway in human disease. J Immunol 2006;176:1305–10.
3. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nat Rev Immunol 2009:9:729–40.
4. Kopp A, Hebecker M, Svobodová E, Józsi M. Factor H: a comple- ment regulator in health and disease, and a mediator of cellular interactions. Biomolecules 2012:2:46–75.
5. Makou E, Herbert AP, Barlow PN. Functional anatomy of comple- ment factor H. Biochemistry 2013;52:3949–62.
6. Blaum BS, Hannan JP, Herbert AP, Kavanagh D, Uhrín D, Stehle T. Structural basis for sialic acid-mediated self-recognition by complement factor H. Nat Chem Biol 2015:11:77–82.
7. Józsi M, Meri S. Factor H-related proteins. Methods Mol Biol 2014:1100:225–36.
8. Goicoechea de Jorge E, Caesar J, Malik TH, Patel M, Colledge M, Johnson S, et al. Dimerization of complement factor H-related proteins modulates complement activation in vivo. Proc Natl Acad Sci USA 2013:110:4685–90.
9. Tortajada A, Yébenes H, Abarrategui-Garrido C, Anter J, García- Fernández JM, Martínez-Barricarte R, et al. C3 glomerulopathy- associated CFHR1 mutation alters FHR oligomerization and complement regulation. J Clin Invest 2013:123:2434–46.
10. Chen Q, Wiesener M, Eberhardt HU, Hartmann A, Uzonyi B, Kirschfink M, et al. Complement factor H-related hybrid protein deregulates complement in dense deposit disease. J Clin Invest 2014:124:45–55.

11. Ferreira VP, Pangburn M, Cortés C. Complement control protein factor H: the good, the bad, and the inadequate. Mol Immunol 2010:47:2187–97.
12. Sculier JP, Coune A, Brassinne C, Laduron C, Atassi G, Ruysschaert JM, et al. 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;4:789–97.
13. Boyajyan A, Ghazaryan H, Stepanyan A, Zakharyan R. Genetic polymorphisms of complement factor H in schizophrenia and ischemic stroke. Mol Immunol 2013;56:294.
14. Lieberman P, Siegle R, Treadwell G. Radiocontrast reactions. Clin Rev Allergy 1986:4:229–45.
15. Lieberman P. Anaphylactoid reactions to radiocontrast materia. Clin Rev Allergy 1991;9:319–38.
16. Szebeni J. Complement activation-related pseudoallergy caused by liposomes, micellar carriers of intravenous drugs and radiocontrast agents. Crit Rev Ther Drug Carr Syst 2001;18:567–606.
17. Szebeni J. Hypersensitivity reactions to radiocontrast media: the role of complement activation. Curr Allergy Asthma Rep 2004;4:25–30.
18. Szebeni J, Kádas J, Lázár J, Takács L, Kurucz I. Plasma proteome profiling with monoclonal antibody libraries: a pilot biomarker analysis for nanomedicine-induced complement activation. Adv Nanopart 2013;2:133–44.
19. 19.Kuznetsova NR, Vodovozova EL. Differential binding of plasma proteins by liposomes loaded with lipophilic prodrugs of metho-trexate and melphalan in the bilayer. Russian in Biokhimiya 2014;79:797–804.
20. De Paula PF, Barbosa JE, Junior PR, Ferriani, VP, Latorre MR, Nudelman V, et al. Ontogeny of complement regulatory proteins concentrations of factor H, factor I, C4b-binding protein, prop- erdin and vitronectin in healthy children of different ages and in adults. Scand J Immunol 2003;58:572–7.
21. Hakobyan S, Harris CL, Tortajada A, Goicochea de Jorge E, García-Layana A. Measurement of factor H variants in plasma using variant-specific monoclonal antibodies: Application to assessing risk of age-related macular degeneration. Invest Ophthalmol Vis Sci 2008;49:1983–90.

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Infusion Reactions Associated with the Medical Application of Monoclonal Antibodies: The Role of Complement Activation and Possibility of Inhibition by Factor H

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Abstract

Human application of monoclonal antibodies (mAbs), enzymes, as well as contrast media and many other particulate drugs and agents referred to as “nanomedicines”, can initiate pseudoallergic hypersensitivity reactions, also known as infusion reactions. These may in part be mediated by the activation of the complement system, a major humoral defense system of innate immunity. In this review, we provide a brief outline of complement activation-related pseudoallergy (CARPA) in general, and then focus on the reactions caused by mAb therapy. Because the alternative pathway of complement activation may amplify such adverse reactions, we highlight the potential use of complement factor H as an inhibitor of CARPA.

Keywords: CARPA; complement; complement activation; factor H; hypersensitivity; infusion reaction; monoclonal antibody therapy; pseudoallergic reaction

Introduction

Monoclonal Antibodies and Hypersensitivity Reactions

Monoclonal antibodies (mAbs) are made by identical immune cells that are all clones of a unique parent B cell, and are widely used both in basic research and the therapy of various diseases. For the latter purpose, one of the main goals of scientists became to create "fully" human products to reduce the side effects of humanized or chimeric therapeutic antibodies. These side effects include the induction of hypersensitivity reactions (HSRs), also known as infusion reactions (IRs) [1]. A selected list of anticancer and anti-inflammatory mAbs that cause such HSRs with various incidence and severity is shown in Table 1 [2–6].

Table 1. Information on hypersensitivity reactions to marketed monoclonal antibodies

Brand Name (Manufacturer)	INN, Isotype (Target Antigen)	Indication	Incidence	Symptoms
Anticancer use				
Avastin (Genentec, Roc San Francisco, CA, USA/Basel Switzerland)	bevacizumab, humanized IgG1 (VEGF-A)	combination chemotherapy of metastatic colon, lung, and kidney cancer, and glioblastoma	<3%, severe: 0.2%	chest pain, diaphoresis, headache, hypertension, neurologic signs and symptoms, oxygen desaturation, rigors, wheezing
Campath (Genzyme Cambridge, MA, USA)	alemtuzumab–IH, humanized IgG1κ (CD52 on T and B cells)	B cell chronic lymphocytic leukemia (B-CLL)	4–7%	bronchospasm, chills, dyspnea, emesis, fever, hypotension, nausea, pyrexia, rash, rigors, tachycardia, urticaria
Erbitux (Brystol-Myers Squibb, New York, NY, USA, Eli Lilly, Indianapolis, IN, USA)	cetuximab, chimeric IgG1κ (EGFR)	metastatic colorectal cancer, head and neck cancer, squamous cell carcinomas	<3%, fatal <0.1%	anaphylaxis, angioedema, bronchospasm, cardiac arrest, chills, dizziness, dyspnea, fever, hoarseness, hypotension, pruritus, rash, rigor, stridor, urticaria, wheezing
Herceptin (Genentech, San Francisco, CA, USA)	trastuzumab, humanized IgG1κ (EGFR receptor 2, HER2/neu/erbB2)	metastatic breast and gastric cancer	<1%	asthenia, bronchospasm, chills, death within hours, dizziness, dyspnea, further pulmonary complications, headache, hypotension, hypoxia, nausea, pain, rash, severe hypotension, vomiting
Rituxan (Genentech, San Francisco, CA, USA)	rituximab, chimeric IgG1κ (CD20 on B cells)	B cell leukemias, rheumatoid arthritis and non-Hodgkin's B-cell lymphoma	>80%, severe: <10%	ARDS, bronchospasm, cardiogenic shock, flushing, hypotension, hypoxia, itching, myocardial infarction, pain (at the site of the tumor), pulmonary infiltrates, runny nose, swelling of the tongue or throat,

				ventricular fibrillation, vomiting
Anti-inflammatory use				
Remicade (Janssen Biotech Inc., Horsham, PA., USA)	infliximab, chimeric IgG1κ (TNF alpha)	Crohn's disease, rheumatoid arthritis, spondylitis ankylopoetica, arthritis psoriatica, ulcerative colitis	18%	bronchospasm, laryngeal edema, pharyngeal edema, dyspnea, hypotension, urticaria, serum sickness-like reactions
Xolair (Genentech, San Francisco, CA, USA)	omalizumab, humanized IgG4 (IgE)	atopia, asthma	39%, Severe: 0.2%	anaphylaxis, bronchospasm, hypotension, syncope, urticaria, and/or angioedema of the throat or tongue, delayed anaphylaxis (with onset two to 24 h or even longer) beyond one year after beginning regularly administered treatment

INN: international nonproprietary names. ARDS: acute respiratory distress syndrom

HSRs have been traditionally categorized in four groups, from I to IV, according to Coombs and Gell. This concept defined Type I reactions as IgE-mediated acute reactions, while the rest of the categories included subacute or chronic immune changes triggered or mediated by IgG, immune complexes, or lymphocytes [7]. However, it has increasingly been recognized that a substantial portion of acute allergic reactions, whose symptoms fit in Coombs and Gell's Type I category, are actually not initiated or mediated by pre-existing IgE antibodies. These reactions are known to be "pseudoallergic" or "anaphylactoid". There are estimates that pseudoallergy may represent as high as 77% of all immune-mediated immediate HSRs [8], implying hundreds of thousands of reactions and numerous fatalities every year [9]. Many of these reactions involve the activation of the complement system, an essential humoral arm of innate immunity. Complement activation-related pseudoallergy (CARPA) is linked to adverse events evoked by several liposomal and micellar formulations, nanoparticles, radiocontrast agents, and therapeutic antibodies [9].

Intravenous application of numerous drugs and medical agents, including therapeutic mAbs, enzymes, radiocontrast media, and many other particulate drugs with physical size in the upper nano (10−8–10−7 m) dimension (nanomedicines), can elicit HSRs with symptoms listed in Table 2.

Table 2. Symptoms of pseudoallergy. The most life-threatening symptoms are highlighted in bold [10].

Cardiovascular	Broncho-Pulmonary	Hematological	Mucocutaneous	Gastrointestinal	Neuro-Psycho-Somatic	Systemic
Angioedema	Apnea	Granulopenia	Cyanosis	Bloating	Back pain	Chills
Arrhythmia	Bronchospasm	Leukopenia	Erythmea	Cramping	Chest pain	Diaphoresis
Cardiogenic shock	Coughing	Lymphopenia	Flushing	Diarrhea	Chest tightness	Feeling of warmth
Edema	Dyspnea	Rebound leukocytosis	Nasal congestion	Metallic taste	Confusion	Fever
Hypertension	Hoarsness	Rebound granulocytosis	Rash	Nausea	Dizziness	Loss of consciousness
Hypotension	Hyperventillation	Trombocytopenia	Rhinitis	Vomiting	Feeling of imminent death	Rigors
Hypoxia	Laryngospasm		Swelling		Fright	Sweating
Myocardial infarction	Respiratory distress		Tearing		Headache	Wheezing
Tachycardia	Shortness of breath		Urticaria		Panic	
Ventricular fibrillation	Sneezing					
Syncope	Stridor					

The Consequences of Complement Activation for the Activator and the Host

One of the major tasks of the complement system is to mark and dispose of potentially dangerous particles, such as pathogenic microbes and altered host cells. This is achieved by targeted activation on foreign surfaces as well as on modified host targets, such as apoptotic cells. The classical pathway is activated by immunoglobulins bound to their target antigens, and the classical and lectin complement pathways are activated upon the recognition of certain molecular patterns associated with microbes or altered self, while the alternative pathway is activated constantly at a low rate and in an indiscriminative manner [11]. The activation can result in the deposition of opsonic molecules on the target cells or particles, thus labeling them for phagocytosis, in addition to (if not inhibited) allowing the initiation of the terminal pathway that may generate lytic complexes in the target cell's membrane. The three pathways merge at the activation of the central C3 molecule, which is cleaved into the anaphylatoxin and inflammatory mediator C3a and the larger, opsonic fragment C3b. C3b feeds back to the

alternative pathway because it is part of the enzyme complex that cleaves additional C3 molecules. Thus, the alternative pathway can amplify complement activation initiated by any of the three pathways. Importantly, complement regulators expressed in body fluids and on cell surfaces protect the host from bystander damage [11].

Complement activation by liposomes can easily be rationalized on the basis of their resemblance to pathogenic viruses. In fact, both are phospholipid-coated vesicles in the same size range (60–200 nm), with the difference being that liposomes do not express surface proteins as viruses do. In the case of viruses, some of these surface proteins inhibit complement activation just as complement receptor type 1 (CR1), decay accelerating factor (DAF), and membrane cofactor protein (MCP) do on the surface of host blood cells and other cells. One may therefore conclude that liposomal nanomedicines activate complement because the immune system considers them as pathogenic viruses, and liposomes do not have a shield that protects them against complement attack [12]. The mechanism of complement activation by smaller nanoparticles (d < 10 nm), such as PEGylated polyethylene-imine polymers (PEG is polyethylene glycol) [13] or micelles formed from Cremophor EL (CrEL) and other polyethoxylated surfactants (PS-80 and PS-20, also known as Tween-20 and Tween-80) [14] is more difficult to explain. In those cases, complement activation may involve unconventional direct interaction with complement proteins, or, as it was suggested for CrEL, prior interaction with plasma lipoproteins that can lead to the formation of large(r) aggregates [9].

Furthermore, it is already shown in vitro that the aggregation of proteins during the preparation of mAbs can induce the activation of human monocyte-derived dendritic cells as well as T cell responses [15]. Complement activation is also possible in such conditions.

Therapeutic mAbs, Complement Activation, and CARPA

Antibodies are well known to activate the classical complement pathway upon binding to their target antigen, which allows for the binding of C1q, the recognition molecule of the activation initiator C1 complex, to the Fc part of the antibodies. Therapeutic mAbs may exploit this feature and can be engineered to enhance the effectiveness of the treatment while circumventing certain (e.g., Fc-receptor-mediated) adverse effects [16,17].

The role that complement plays in mAb therapy is exemplified well by the prototypic mAb rituximab. Rituximab, a murine-human chimera type anti-CD20, has been used since 1997 in clinical practice to treat malignant and autoimmune disorders related to the disfunction of B cells [18,19]. Besides the direct downregulation of CD20-related cell functions, both complement-dependent and complement-independent immune reactions participate in the

elimination of CD20 highly positive B cells (Figure 1). Complement-dependent mechanisms include complement-dependent cytotoxicity (CDC), initiated upon C1q binding, through the classical complement activation cascade [20], and complement-enhanced antibody-dependent cell-mediated phagocytosis (ADCP). The most important complement-independent mechanism is antibody-dependent cell-mediated cytotoxicity (ADCC), which is performed mainly by NK cells (and macrophages). Programmed cell death (PCD) seems to be less important in the case of rituximab, but it may have more prominent role in the action of Type II-anti-CD20 antibodies, like tositumomab and GA101 [18,19]. However, it is likely that the complement-activating capacity of rituximab is also responsible for the high frequency of CARPA associated with this mAb [21].

Human IgG1 and IgG3 are particularly effective at fixing complement to the target cell surface, and many of the currently approved therapeutic mAbs, like rituximab, are indeed of the IgG1 isotype. A variety of cell-based assays have demonstrated the ability of mAbs to recruit complement components in vitro, but the efficiency of CDC to kill tumor cells in vivo is less clear, particularly for solid tumors, in part because tumor cells themselves express membrane-bound complement regulators as well as the soluble regulator factor H [22–24]. Since most of these mAbs work against cancer cells with the help of complement activation, a clear distinction has to be made between complement activation on the target cell surface with the help of the cell-bound mAb (i.e., CDC) and adverse hypersensitivity reaction related to complement activation in serum caused by the therapeutic antibody itself. This means that the same mechanisms are involved in the beneficial effects and hypersensitivity.

All currently available or publicly known mAbs can be considered to be potentially direct immunogens, as their molecular size is large enough and their structure is different from endogenous proteins. Despite current efforts to produce highly humanized or “human-like” mAbs, immunogenicity is not yet totally eradicated. Treatment of human patients with mAbs can be associated with the development of specific antibodies against these therapeutic antibodies (anti-drug antibodies, ADAs). These neutralizing ADAs can block the biological activity of the drug either by binding directly to the epitope(s) within their active site, or by steric hindrance due to binding to epitope(s) in close proximity to the active site. The presence of neutralizing ADAs may not result in adverse clinical effect, except that it decreases the efficacy of the therapeutic mAb, requiring its administration at higher doses. Furthermore, the presence of specific ADAs against mAbs can be associated in some cases with hypersensitivity

reactions identical to the CARPA phenomenon delineated above for the case of liposomes and other nano-pharmaceuticals. The rare anaphylactic reactions associated with mAbs including cetuximab, infliximab, or basiliximab represent typical CARPA [25].

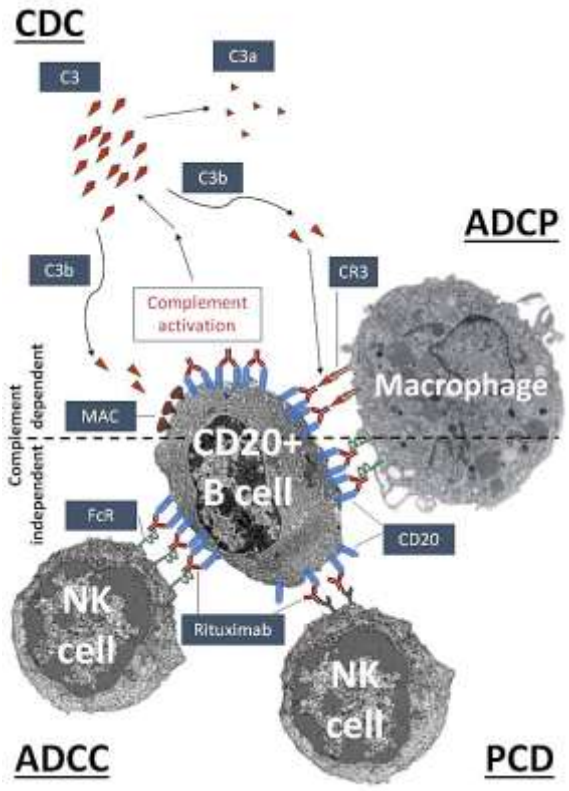

Figure 1. Complement activation as an essential mechanism of the therapeutic action of rituximab, an anti-CD20 antibody. Rituximab recognizes CD20 on the surface of pre- and mature B cells. After binding, the complement activation cascade is initiated by the classical pathway leading to the cleavage of C3 into C3a and C3b. C3b can cause complement-dependent cytotoxicity (CDC) by promoting the assembly of the membrane attack complex (MAC), while complement receptors on phagocytic cells, such as complement receptor type 3 (CR3) on macrophages, can mediate complement-enhanced antibody-dependent cell-mediated phagocytosis (ADCP). Surface-bound rituximab can trigger NK cells and macrophages by complement-independent mechanisms, via antibody-dependent cell-mediated cytotoxicity (ADCC), ADCP and, to a lesser degree, the induction of programmed cell death (PCD).

True allergic reactions, which are mediated by anti-drug IgE, require prior exposure to the mAb and, consequently, do not occur on the first infusion, except in rare cases where patients have

pre-existing antibodies that cross-react with the drug. However, pseudoallergic reactions (IgE-independent reactions possibly mediated by direct immune cell and complement activation) and cytokine release syndrome (CRS) both occur primarily on the first infusion of the drug, although they can also occur on subsequent administrations. The symptoms of all three types of immunologically-mediated infusion reactions (IRs) overlap, making it difficult to identify the cause without additional laboratory work [26].

Rituximab and trastuzumab induce the highest incidence of IRs. In general, the incidence of mAb-induced IRs varies from ~15–20% for cetuximab (including 3% more severe, grade 3, and life threatening, grade 4 reactions) and 40% for trastuzumab first infusion (<1% grades 3–4) to 77% for rituximab first infusion (10% grades 3–4). Even after the fourth infusion, 30% of cancer patients react to rituximab, and the incidence of IRs remains 14% after the eighth infusion. Approximately 80% of fatal reactions occur after the first rituximab infusion. The incidence of IRs to the humanized mAb bevacizumab and the fully humanized panitumumab is significantly lower [27].

Thrombocytopenia, neutropenia, and anemia can occur in some patients treated with mAbs as part of anticancer immunotherapy, but the mechanisms of these potentially severe side effects frequently remain unexplored. Interestingly, these symptoms are also characteristic of liposome-induced CARPA. Late-onset neutropenia, especially after rituximab treatment, has been examined in a growing number of reports; however, with each of the three cytopenias seen during mAb therapy, it is frequently unclear whether the depletion of cells is due to an immunological mechanism. Type III hypersensitivities, such as serum sickness-like reactions and vasculitis, are also known to occur in response to mAbs. Some pulmonary events, including mAb-induced lung diseases, are hypersensitivity reactions that result from the interaction of the drug with the immune system and involve drug-specific antibodies or T cells [2].

Although it remains to be shown in humans, it is hypothesized that mAbs could stimulate anti-mAb IgGs bound to Fc-gamma-receptors on macrophages, basophils, and neutrophils, triggering the release of platelet-activating factor, as shown in the mouse model of IgG-dependent anaphylaxis [28]. In addition, the complement system could be activated by the formation of large immune complexes, thereby generating anaphylatoxins (C3a and C5a). It is also important to point out that patients with anti-infliximab IgGs are at increased risk of immediate HSRs compared with patients without such antibodies [1]. Thus, in addition to the preferred complement activation induced by the binding of therapeutic mAbs to their targets, complement activation can also arise as a consequence of the binding of naturally forming

ADAs against the therapeutic mAbs. The molecular background of mAb-induced CARPA is yet to be studied in more detail.

Potential Role of Factor H in Mitigating Complement Activation

The use of natural or engineered complement inhibitors may represent an attractive way to prevent CARPA-mediated HSRs. Early approaches used the complement-regulatory domains of the natural complement inhibitor CR1 linked to a myristoyl group that mediated incorporation in liposomal membranes [29]. A recent study suggested that factor H could be also employed to reduce or eliminate complement activation triggered by liposomes, micelles, or therapeutic mAbs [30]. Factor H is the main soluble inhibitor of the alternative pathway and the amplification loop of complement [31,32]. It was shown that liposomal Amphotericin B, CrEL, and rituximab caused less complement activation in serum in vitro when factor H was added to the serum in excess, as compared with the serum without exogenous factor H [30]. Moreover, the artificial inhibitor, recombinant mini-factor H [33], which unites the N-terminal complement-regulatory domains and the C-terminal host surface recognition domains of the natural molecule, was even more effective in inhibiting such complement activation compared with factor H [30]. These data suggest that factor H-based complement inhibition could be a viable strategy to prevent or mitigate CARPA induced by nanomedicines, including therapeutic mAbs.

Conclusion and Outlook

The prevention of IRs induced by mAbs can be addressed the same way as the prevention of similar adverse reactions occurring upon nanomedicine treatments. The surface modification of liposomes and other therapeutic proteins can lead to prevention of the aggregation of these agents and reduction of immunogenicity and antigenicity. Recently, more and more antibodies and, predominantly, antibody fragments designed for therapeutic purposes use the covalent attachment of polyethylene glycol (PEG). PEGylation generally prolongs the half-life in the circulation and prevents the immunogenicity of many liposomal drugs and mAb molecules [34]. However, in some cases the generation of an IR event could be connected to the presence of PEGylation on the surfaces of liposomes. The formation of anti-PEG IgMs against PEG molecules on liposomes are observed in CARPA studies with animal models [35].

Another possible approach is the administration of complement inhibitors together with the therapeutic agents to reduce the chance of a possible adverse reaction. Even though this could be a good option as a prevention measure, most patients may not even need such an action if

they are not prone to IRs, and this approach would just elevate the costs of the therapies. The best scenario would be to pre-screen each patient for proneness to any adverse reaction, using an in vitro test that could predict from a blood sample if any CARPA event could arise during introduction of a therapeutic agent, such as mAbs.

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References

1. Picard, M.; Galvão, V.R. Current Knowledge and Management of Hypersensitivity Reactions to Monoclonal Antibodies. J. Allergy Clin. Immunol. Pract. 2017, 5, 600–609.
2. Baldo, B.A. Adverse events to monoclonal antibodies used for cancer therapy: Focus on hypersensitivity responses. Oncoimmunology 2013, 2, e26333.
3. Hong, D.I.; Bankova, L.; Cahill, K.N.; Kyin, T.; Castells, M.C. Allergy to monoclonal antibodies: Cutting-edge desensitization methods for cutting-edge therapies. Expert Rev. Clin. Immunol. 2012, 8, 43–52.
4. Choueiri, T.K.; Mayer, E.L.; Je, Y.; Rosenberg, J.E.; Nguyen, P.L.; Azzi, G.R.; Bellmunt, J.; Burstein, H.J.; Schutz, F.A. Congestive Heart Failure Risk in Patients With Breast Cancer Treated With Bevacizumab. J. Clin. Oncol. 2011, 29, 632–638.
5. Keating, M.J.; Flinn, I.; Jain, V.; Binet, J.L.; Hillmen, P.; Byrd, J.; Albitar, M.; Brettman, L.; Santabarbara, P.; Wacker, B.; et al. Therapeutic role of alemtuzumab (Campath-1H) in patients who have failed fludarabine: Results of a large international study. Blood 2002, 99, 3554–3561.

6. Kimby, E. Tolerability and safety of rituximab (MabThera®). Cancer Treat. Rev. 2005, 31, 456–473.
7. Coombs, R.R.A.; Gell, P.G.H. Classification of allergic reactions responsible for drug hypersensitivity reactions. In Clinical Aspects of Immunology; Coombs, R.R.A., Gell, P.G.H., Eds.; Davis: Philadelphia, PA, US, 1968; pp. 575–596.
8. Demoly, P.; Lebel, B.; Messaad, D.; Sahla, H.; Rongier, M.; Daurès, J.P.; Godard, P.; Bousquet, J. Predictive capacity of histamine release for the diagnosis of drug allergy. Allergy 1999, 54, 500–506.
9. Szebeni, J. Complement activation-related pseudoallergy caused by liposomes, micellar carriers of intravenous drugs, and radiocontrast agents. Crit. Rev. Ther. Drug Carrier Syst. 2001, 18, 567–606.
10. Szebeni, J. Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals. Mol. Immunol. 2014, 61, 163–173.
11. Ricklin, D.; Hajishengallis, G.; Yang, K.; Lambris, J.D. Complement: A key system for immune surveillance and homeostasis. Nat. Immunol. 2010, 11, 785–797.
12. Szebeni, J.; Muggia, F.; Gabizon, A.; Barenholz, Y. Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: Prediction and prevention. Adv. Drug Deliv. Rev. 2011, 63, 1020–1030.
13. Merkel, O.M.; 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.
14. Weiszhar, Z.; Czúcz, 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.
15. Rombach-Riegraf, V.; Karle, A.C.; Wolf, B.; Sordé, L.; Koepke, S.; Gottlieb, S.; Krieg, J.; Djidja, M.C.; Baban, A.; Spindeldreher, S.; et al. Aggregation of human recombinant monoclonal antibodies influences the capacity of dendritic cells to stimulate adaptive T-cell responses in vitro. PLoS ONE 2014, 9, e86322.
16. Cook, E.M.; Lindorfer, M.A.; van der Horst, H.; Oostindie, S.; Beurskens, F.J.; Schuurman, J.; Zent, C.S.; Burack, R.; Parren, P.W.; Taylor, R.P. Antibodies That Efficiently Form Hexamers upon Antigen Binding Can Induce Complement-Dependent Cytotoxicity under Complement-Limiting Conditions. J. Immunol. 2016, 197, 1762–1775.

17. Lee, C.H.; Romain, G.; Yan, W.; Watanabe, M.; Charab, W.; Todorova, B.; Lee, J.; Triplett, K.; Donkor, M.; Lungu, O.I.; et al. IgG Fc domains that bind C1q but not effector Fcγ receptors delineate the importance of complement-mediated effector functions. Nat. Immunol. 2017, 18, 889–898.
18. Lim, S.H.; Beers, S.A.; French, R.R.; Johnson, P.W.; Glennie, M.J.; Cragg, M.S. Anti-CD20 monoclonal antibodies: Historical and future perspectives. Haematologica 2010, 95, 135–143.
19. Boross, P.; Leusen, J.H. Mechanisms of action of CD20 antibodies. Am. J. Cancer Res. 2012, 2, 676–690.
20. Zhou, X.; Hu, W.; Qin, X. The role of complement in the mechanism of action of rituximab for B-cell lymphoma: Implications for therapy. Oncologist 2008, 13, 954–966.
21. Van der Kolk, L.E.; Grillo-Lopez, A.J.; Baars, J.W.; Hack, C.E.; van Oers, M.H. Complement activation plays a key role in the side-effects of rituximab treatment. Br. J. Haematol. 2001, 115, 807–811.
22. Junnikkala, S.; Jokiranta, T.S.; Friese, M.A.; Jarva, H.; Zipfel, P.F.; Meri, S. Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. J. Immunol. 2000, 164, 6075–6081.
23. Ajona, D.; Castaño, Z.; Garayoa, M.; Zudaire, E.; Pajares, M.J.; Martinez, A.; Cuttitta, F.; Montuenga, L.M.; Pio, R. Expression of complement factor H by lung cancer cells: Effects on the activation of the alternative pathway of complement. Cancer Res. 2004, 64, 6310–6318.
24. Rogers, L.M.; Veeramani, S; Weiner, G.J. Complement in Monoclonal Antibody Therapy of Cancer. Immunol. Res. 2014, 59, 203–210.
25. Descotes, J. Immunotoxicity of monoclonal antibodies. mAbs 2009, 1, 104–111.
26. Brennan, F.R.; Morton, L.D.; Spindeldreher, S.; Kiessling, A.; Allenspach, R.; Hey, A.; Muller, P.Y.; Frings, W.; Sims, J. Safety and immunotoxicity assessment of immunomodulatory monoclonal antibodies. mAbs 2010, 2, 233–255.
27. Chung, C.H. Managing premedications and the risk for reactions to infusional monoclonal antibody therapy. Oncologist 2008, 13, 725–732.
28. Strait, R.T.; Morris, S.C.; Yang, M.; Qu, X.W.; Finkelman, F.D. Pathways of anaphylaxis in the mouse. J. Allergy Clin. Immunol. 2002, 109, 658–668.
29. Smith, G.P.; Smith, R.A. Membrane-targeted complement inhibitors. Mol. Immunol. 2011, 38, 249–255.

30. Mészáros, T.; Csincsi, Á.I.; Uzonyi, B.; Hebecker, M.; Fülöp, T.G.; Erdei, A.; Szebeni, J.; Józsi, M. Factor H inhibits complement activation induced by liposomal and micellar drugs and the therapeutic antibody rituximab in vitro. Nanomedicine 2016, 12, 1023–1031.
31. Kopp, A.; Hebecker, M.; Svobodová, E.; Józsi, M. Factor H: A complement regulator in health and disease, and a mediator of cellular interactions. Biomolecules 2012, 2, 46–75.
32. Parente, R.; Clark, S.J.; Inforzato, A.; Day, A.J. Complement factor H in host defense and immune evasion. Cell. Mol. Life Sci. 2017, 74, 1605–1624.
33. Hebecker, M.; Alba-Domínguez, M.; Roumenina, L.T.; Reuter, S.; Hyvärinen, S.; Dragon-Durey, M.A.; Jokiranta, T.S.; Sánchez-Corral, P.; Józsi, M. An engineered construct combining complement regulatory and surface-recognition domains represents a minimal-size functional factor H. J. Immunol. 2013, 191, 912–921.
34. Milla, P.; Dosio, F.; Cattel, L. PEGylation of proteins and liposomes: A powerful and flexible strategy to improve the drug delivery. Curr. Drug. Metab. 2012, 13, 105–119.
35. Hashimoto, Y.; Shimizu, T.; Abu Lila, A.S.; Ishida, T.; Kiwada, H. Relationship between the concentration of anti-polyethylene glycol (PEG) immunoglobulin M (IgM) and the intensity of the accelerated blood clearance (ABC) phenomenon against PEGylated liposomes in mice. Biol. Pharm. Bull. 2015, 38, 417–424.

7
Summarizing discussion and perspectives

Nanomedicines and complement activation

The applications of nanomedicine are wide-spread: as drug carriers (e.g. Abraxane, Doxil, Rapamune), as diagnostic agents (several nanoparticles like quantum dots are used in imaging as contrast agents), as nanosensors (like the novel lab-on-a-chip technologies), in physical therapy applications (such as blood purification with magnetic nanoparticles and tissue engineering), and for several analytical research purposes. Combining the science of nanostructures with the power of pharmacologically active molecules and diagnostic agents can yield valuable applications and products. Clinical research on drug carrier nanomedicines has particularly addressed applications for the diagnosis and treatment of cancer, HIV, auto-immune diseases, and infections. The possibility to change the surface of nanomedicine carriers carrying different active pharmaceutical ingredients (API) by interfacing them with designable biological molecules and structures, such as modification with targeting ligands, is a valuable asset but did not yield clear clinical benefit yet.

In **Chapter 1** the connection of such nanomedicines with the induction of hypersensitivity reactions upon their IV administration, called complement activation-related pseudoallergy (CARPA), is introduced. Intravenous injection of a variety of nanomedicinal (liposomal, micellar, polymer-conjugated) and protein-based (antibodies, enzymes) drugs can lead to hypersensitivity reactions (HSRs), also known as infusion or anaphylactoid reactions. The molecular mechanism behind and severity of these HSRs may alter from case to case, and in many cases the major cause, or contributing factor is activation of the complement (C) system. CARPA, a non-IgE-mediated allergy syndrome, is up to now unpredictable and was occasionally lethal. Certain nanomedicines and other agents activate C through both the classical and the alternative pathways, giving rise to C3a and C5a anaphylatoxins that trigger mast cells, basophils, macrophages and other anaphylatoxin receptor-expressing cells for secretory responses that underly HSRs. [1]

This thesis work has been centered around PEGylated liposomes and SPIONs (superparamagnetic iron oxide nanoparticles) as successful examples of marketed nanomaterials. In the last twenty years, the most successful nanomedicine family within the broad field of nanomedicine so far are indeed liposomes. [2] These nanocarriers are widely used and available on the market in several forms. SPIONS are nanocarriers used for imaging

purposes, and just as for liposomal drug carrier products, their unwanted C activating ability should not be neglected. Another agent used in therapeutics that can induce C related HSRs similar to nanomedicines are monoclonal antibodies (mABs) with or without PEGylation. These biomolecules are also quite novel, preferred and widely used in the medical field with lots of possibilities. Every detail in the molecular background how they activate the C system is not perfectly understood, however the side effects they induce are usually similar to HSRs generated by the intravenous use of particulate nanomedicines. [3,4]

Furthermore, this thesis also focuses on thromboxane (TxB2) changes during CARPA. TxB2, the stable metabolite of vasoactive mediator, TxA2, serves as a biomarker of HSRs. [5-7] The review in Chapter 4 goes in detail how the rise of its blood level parallels the hemodynamic changes produced by C activation by a nanomedicine. Complement factor H (FH), a natural C activation inhibitor in blood, was theorized to be a good candidate to work against CARPA. [8] As described above, PEGylated mABs are also able to produce HSRs with symptoms typical of CARPA. [9]

One aim of this thesis work was to find options to reduce the effects of C activation-related HSRs or find options to predict and prevent them. As pigs serve as a sensitive in vivo model of CARPA, PEGylated-liposomal prednisolone (a nanomedicine candidate that reached clinical trials) was tested in this model for studying the effect of altering the infusion protocol as a way to reduce or even eliminate the risk of HSRs (Chapter 2). Then the thesis elaborates in Chapter 3 on experiments carried out to test the correlation between HSRs caused by C activation induced by low-molecular-weight dextran-coated superparamagnetic iron-oxides (SPIONs) and the physiochemical features of the latter nanoparticles. Chapters 4 and 5 go into details of finding possible biomarkers for CARPA prediction via analyzing and reviewing molecules involved in C activation. In this context, the role of thromboxane (TxA2) was emphasized on the basis of studies in the porcine CARPA model. Furthermore, the role of complement factor H (FH) reduction in HSRs induced by mAbs is detailed with particular focus on the question if this biomarker could be used to predict CARPA caused by mABs.

The summaries and discussion of the results of each experimental chapter follow below.

PLP in the porcine CARPA model: impact of infusion protocol

PLP investigated in **Chapter 2** have been developed to treat diseases with a strong chronic inflammation component such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis. PEGylation is a widespread method to prolong the circulation time of nanocarriers in the circulation to improve their targeting capability. However, an unresolved problem with such PEGylated nanomedicines is their unwanted immune recognition resulting in non-IgE mediated, pseudoallergic hypersensitivity reactions. These adverse reactions occur in certain patients causing symptoms with varying severity, resulting in a few cases even in a fatal pseudo-anaphylactic shock. Evidently, finding possibilities to prevent such reactions are of utmost importance. With the help of the porcine CARPA model, it was possible to assess infusion reactions very similar to those seen in patients when infusing PLP. Several parameters were monitored, including hemodynamic changes (heart rate, systemic arterial pressure, pulmonary arterial pressure, pCO2, SpO2), blood cell count analysis and TxB2 changes measured with ELISA. The adverse responses could be significantly reduced or eliminated by slowing down the infusion rate of PLP, shown in experiments showing that a 3h 3-step dosage escalation protocol was the least reactogenic. Interestingly, the presence of prednisolone phosphate inside the aqueous interior of the liposomes, had no inhibitory activity on the PLP-induced HSRs, indicating that the PLP remain intact and do not leak the immunosuppressive drug in the bloodstream. Thus, based on this and other previously observed similar HSR events including PEGylated carriers, most likely the PEG coating is the main trigger for such events. A possible mechanism for the risk reduction provided by slow infusion is that the blood levels of anaphylatoxins (complement peptide fragments playing a causal role in HSRs) remain below the threshold concentration required to trigger HSRs, thus avoiding the occurrence of CARPA.

SPIONS in the CARPA model: impact of physicochemical properties

Yet another prominent example of nanoparticulate nanomedicine used in imaging and drug delivery applications imaging is the use SPIONs. Also iron-containing drugs and contrast media can cause HSRs as shown in several patients showing symptoms of CARPA. It is important to point out that, though the HSR symptoms are identical, the C activation and involved biological pathways leading to such adverse reactions differ from one nanomedicine to another, and that the exact mechanism in case of SPIONs may differ from the C activation mechanism in case of PEGylated PLP surfaces. In **Chapter 3**, we determined C activation induced by 6 different types of SPIONs in human serum samples that in previous experiments gave strong C activation when the marketed nanomedicine product Doxil (Caelyx, PEG-liposomal doxorubicin) was

added. Strikingly, the C activation potential of SPIONs appeared to depend strongly on their physicochemical features regarding iron core composition and surface coating. In fact, we observed that only the dextran-coated SPIONs caused C activation in human serum. When comparing Sinerem with Resovist in follow-up tests in 15 different human sera, we obtained quite variable results but could observe that the former SPION type was a stronger C activator than the latter. Consistent with this in vitro outcome, Sinerem gave dose-dependent CARPA in a pig upon intravenous administration while Resovist did not. These results are in line with Sinerem's withdrawal from the market due to previous HSRs occurring in patients. To find an explanation for this difference between Sinerem and Resovist, we performed dynamic light scattering and nanoparticle tracking analysis experiments. The results obtained with Sinerem show the presence of a much larger number of relatively large (> 100 nm) nanoparticles and a significant fraction of aggregates. This makes Sinerem a more inhomogeneous dispersion compared to Resovist. We suggest that such inhomogeneity correlates with the difference in reactogenicity and presents a considerable safety risk. These observations also highlight the importance of the use of adequate nanoparticle characterization methods during nanomedicine development.

Possible biomarkers of C-related HSRs

Basically, during the last 15 years, to understand the background and origin of CARPA, C activation by nanocarriers and the several intertwining pathways leading to numerous symptoms were studied in vitro and in vivo by several research groups. The use of biomarkers of HSRs were also a main focus in our studies. Biomarkers are measurable indicators of a biological condition or state. In our case these molecules are connected to either C activation or associated amplification processes. In **Chapters 4** and **5**, respectively, the role of thromboxane (TxB2) and complement factor H (FH) in relation to triggering CARPA were discussed. Thromboxane, a vasoconstrictor, is a key biomarker of CARPA. Increased levels in blood and the rise of pulmonary arterial pressure (PAP) proceed hand-in-hand in pigs within minutes after i.v. injection of reactogenic nanoparticles, providing evidence for a close linkage between these processes. Because this ready response is highly reproducible and quantitative, monitoring PAP and early TxB2 changes (beside several other physiological changes) allows the study of the dose-response relationship between drug administration and occurrence of HSRs (see Fig.5 in Chapter 4). Thus, TxB2 is a good biomarker for monitoring the intensity and progress of a C-related HSR. From collected plasma, TxB2 ELISA measurements are always part of the full porcine CARPA evaluation protocol. In pigs, the pulmonary intravascular

macrophages (PIM cells) are held responsible for the dramatic cardiopulmonary changes during the reactions. PIM cells are known to secrete TxA_2 (precursor of TxB2), which is consistent with the claimed primary role of TxA2 in the hemodynamic changes seen in the pig. However, these thromboxane secreting PIM cells are not known to be present in human lungs, so the possible presence of PIM like cells secreting the biomarker in highly reacting patients is still ill-understood. Preliminary clinical information suggests that indomethacin pretreatment is effective in preventing Doxil (liposomal doxorubicin)-induced CARPA in cancer patients.[10] The efficacy of indomethacin, a cyclooxygenase inhibitor, in restricting liposome-induced CARPA in these cancer patients is also consistent with the key role of TxA2 in mediating these reactions, since indomethacin is a potent inhibitor of prostaglandin synthesis by blocking both cyclooxygenases (COX)-1 and COX-2, important players in TxA2 synthesis.

Another potential biomarker, Factor H (FH), a natural inhibitor of C activation, got into focus because of its possible capability to influence the proneness to and the severity of HSRs occurring in patients. FH is a well-known and studied inhibitor of C activation via the alternative pathway, a major factor holding excessive C activation under control. Plasma samples of patients with autoimmune diseases (inflammatory bowel disease and rheumatoid arthritis) collected in two clinical trials in which they were treated with PLP, were analyzed with a commercially available human FH ELISA kit. However, determination of FH levels is complicated by the presence of at least six other related proteins: FHL-1 (Factor H like protein 1) and FHR proteins encoded by five CFHR (complement factor H) genes: FHR1-5 (Factor H related proteins 1-5) in plasma/serum samples. The results did not show any possible correlation between FH level changes and HSR occurrence, most likely because the FH ELISA kit measures not only FH but also other FHRs, that also carry the short consensus repeat (SCR) domains (also termed complement control protein domains, CCPs) in the antigen for which the ELISA was actually developed to detect. This technical problem is currently being addressed by developing a truly FH specific ELISA that can exclude the detection of any other FH-related proteins.

PEGylated monoclonal antibodies and adverse C activation

Not only therapeutic PEGylated liposomes but also PEGylated monoclonal antibodies (mABs) can cause C activation-related HSRs as discussed in **Chapter 6**. More and more therapeutic mABs and antibody fragments are designed with covalent attachment of polyethylene glycol (PEG). PEGylation generally prolongs the half-life in the circulation and reduces the immunogenicity of the mAb molecules just as in the case of PEGylated liposomes. However,

just as with other PEGylated nanomedicines, mABs can unfortunately initiate C activation in an IgE independent way, producing HSRs. Some patients can develop antibodies against these therapeutic PEGylated-mABs (anti-drug antibodies, ADAs). These neutralizing ADAs can block the biological activity of therapeutic antibodies either by binding directly to the epitope(s) within their active site, or by steric hindrance due to binding to epitope(s) in close proximity to the active site. The presence of neutralizing ADAs may not result in adverse clinical effects, but it can decrease the efficacy of the therapeutic mAb, requiring the administration of higher doses. True allergic reactions (mediated by anti-drug IgE) require prior exposure to the therapeutic PEGylated-mAb and, consequently, do not occur at the first infusion, except in rare cases where patients have pre-existing antibodies that cross-react with the drug. On the other hand, pseudoallergic reactions primarily occur at the first time of the administration of the PEGylated-mABs (sometimes they also occur at the next administration). As both types of reactions are infusion reactions with fairly similar symptoms, it is hard to distinguish them without checking the molecular background behind the HSRs. The relevance of C activation-related HSRs induced by therapeutic mABs can be illustrated with Rituximab (a commercialized mAB used in treatments against autoimmune diseases (arthritis, lupus) and cancer treatment (non-Hodgkin lymphoma, leukemia): approximately 80% of the reactions with fatal outcome (11 patients) occurred after its first infusion. Reflecting back to the previous chapter, a recent study suggested that FH could also be employed to reduce or eliminate complement activation triggered by liposomes, micelles, and therapeutic mAbs.[11] As FH is the main soluble inhibitor of the alternative pathway and the amplification loop of complement, it would seem an excellent candidate for decreasing or eliminating the risk of CARPA reactions via administering it at the same time as the infusion of the therapeutic agent starts. It was shown in our study that liposomal Amphotericin B, cremophor EL (CrEL), a formulation vehicle for poor-water-soluble agents, and Rituximab caused less complement activation in serum in vitro when FH was added to the serum in excess, as compared with the serum without exogenous FH.

Conclusions and future perspectives

Clearly, CARPA is a complex phenomenon and understanding of the underlying mechanisms has begun only recently, leaving up to the future to clarify many of its details and find solutions to prevent it or reduce its harmful consequences. What is known that C activation can be the primary trigger, but there are reactions involving other players of the immune system with C activation being a contributing factor. C activation by different nanomedicines and the release of secondary mediators may proceed via different pathways depending on the physicochemical characteristics of these nanoparticles, explaining the diverse intensity and variable types of the CARPA symptoms that can occur in individual patients.

The main objective addressed in this thesis was to investigate the mechanism, prediction and prevention of the risks of C-activated HSRs caused by polymer-coated nanomedicines like PEGylated liposomes, SPIONs and biomolecules like therapeutic PEGylated monoclonal antibodies. From the research outcome, certainly certain theories and ideas can be deducted that could play an important role in future nanomedicine development.

According to the "anaphylatoxin balance" theory, the blood level of anaphylatoxins (C peptide fragments responsible for anaphylaxis), C3a and C5a, is determined by their generation via C activation and subsequent clearance by cellular uptake by macrophages and metabolism by carboxypeptidases in the plasma. When massive anaphylatoxin formation exceeds its clearance, which is timewise much slower, their blood level may rapidly spike to reach a threshold where the allergy mediating cells release their mediators, resulting in HSR. In contrast, slow formation of anaphylatoxins during slow infusion may be coped with by clearance, keeping the concentration of anaphylatoxins below the HSR threshold. This brought us to answering the first question of this thesis, whether the risks of HSRs could be reduced to PEG-liposomal prednisolone by modifying the infusion protocols in pig model. As the porcine CARPA experiment results show in **Chapter 2**, the infusion protocol administering the PEGylated prednisolone containing liposomes with a "slower" 3h long 3-step dosage escalation protocol proved safer regarding cardiovascular reactivity and preventing the induction of CARPA symptoms. Though these protocols may vary for different nanosystems, including PEGylated and non-PEGylated ones with different reactivities, the pig model is instrumental as an 'in vivo tool` to fine-tune the optimal parameters.

As we need to learn from the mistakes of the past, the prevention of unwanted HSRs should in the future already start at an early development stage of nanomedicinal drugs. This is illustrated by the work of **Chapter 3,** Sinerem (SPION withdrawn from the market) was compared with

Resovist (SPION used as MRI contrast agent) regarding C activation potency and reactivity in vivo. The main question brought up in this thesis chapter was whether there is a correlation between the reactogenicity of these PEGylated nanoparticles and their physiochemical features. Sinerem showed stronger C activation (consistent with the reason for its withdrawal from the market due to HSRs occurring in patients) than Resovist. Upon physiochemical characterization of these two SPION types, an increased amount of aggregates could be observed in Sinerem relative to Resovist. Therefore, we advise to use adequate methods for physiochemical characterization of nanomedicines in development, including the formation of aggregates (precipitates) over time, to avoid increased risk for CARPA induction.

The plan to develop methods for analyzing biomarkers of C activation in blood samples of patients (prior to administration of the nanomedicine to check the patients` proneness to HSR) led to our review of the role of the vasoactive mediator thromboxane A2 in CARPA in **Chapter 4**. Interestingly, the consistent observation in many of our preclinical studies over the past decade showed that TxA2 is a key, rate-limiting mediator of hemodynamic changes in CARPA. This observation may therefore represent a major step towards solving the CARPA problem: HSR prevention could be achieved by taking relatively cheap and nontoxic NSAIDs before the administration of the nanomedicine. Another practical benefit of the observation that TxA2 plays a key role that thromboxanes can potentially serve as a quantitative biomarker to indicate the severity of reactions. Another consideration for a biomarker was Factor H, a complement protein that inhibits the amplification of C activation cascades in the system. In **Chapter 5**, FH was planned to be analyzed with a commercially available FH ELISA kit in plasma samples of patients treated with PEGylated liposomes containing prednisolone phosphate. However, the determination of the plasma levels of FH using the kit was problematic: the high levels of FH in the plasma samples suggested that the antibodies used for the ELISA kit are specific to a short consensus repeat in molecular structures that also can be found in FH-like and FH-related proteins. In future studies, an antibody specific to an epitope present only in FH should be developed for measuring the accurate plasma levels of FH.

Occurrence of infusion reactions is not specific to nanopharmaceutical drug delivery systems, similar symptoms can occur due to C activation induced by PEGylated therapeutic monoclonal antibodies as well. In **Chapter 6**, it is shown that PEGylated mABs produce infusion reactions as well, with mild to severe symptoms quite similar to PEG-liposomes induced CARPA reactions. As most of these HSRs occur upon the first administration of the drug (just as with PEG-liposomes), preventive measures should be implemented in the near future. The last main question of the thesis brought forward the option to introduce complement inhibitors, just as

the previously mentioned factor H, prior to administration of the therapeutic PEG-mABs. Just as it was discussed with nanoparticulate medicines, the best scenario would be to pre-screen the patients to proneness to C activation by the agent: in future studies, the right biomarker combinations should be identified to be used in such pre-screening assays.

We anticipate that the use of nanomedicines for therapy and diagnostics will be on the rise and that and research activities will be extended in the near future. Recent research on PEGylated liposomal drug formulations showed the presence of natural anti-PEG IgM antibodies in porcine CARPA experiments, possibly connected to C activation and HSR. For example, rethinking and inventing new PEGylation methods or use of new PEG constructs may be able to eliminate the risks of HSRs later on. With the previously mentioned pre-screening tests using certain biomarkers of C activation, the development of safe nanopharmaceutical products and therapeutic mABs can be improved in the future. However, since the exact molecular and physiological mechanisms underlying C activation may differ from product to product, different product-specific pre-screening tests should be developed. All in all, reducing and preventing the risks of CARPA reactions for patients treated with nanomedicines will help to improve and widen the clinical application possibilities of nanomedicines.

References

1. Szebeni J. Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity.Toxicology. 2005;216(2-3):106-121. doi:10.1016/j.tox.2005.07.023
2. Crommelin DJA, van Hoogevest P, Storm G. The role of liposomes in clinical nanomedicine development. What now? Now what? J Control Release. 2020;318:256-263. doi:10.1016/j.jconrel.2019.12.023
3. Hong D, Sloane DE. Hypersensitivity to monoclonal antibodies used for cancer and inflammatory or connective tissue diseases. Ann Allergy Asthma Immunol. 2019;123(1):35-41. doi:10.1016/j.anai.2019.04.015
4. Picard M, Galvão VR. Current Knowledge and Management of Hypersensitivity Reactions to Monoclonal Antibodies. J Allergy Clin Immunol Pract. 2017;5(3):600-609. doi:10.1016/j.jaip.2016.12.001
5. Szebeni J, Alving CR, Rosivall L, et al. Animal models of complement-mediated

hypersensitivity reactions to liposomes and other lipid-based nanoparticles. J Liposome Res. 2007;17(2):107-117. doi:10.1080/08982100701375118

6. Dézsi L, Mészáros T, Őrfi E, et al. Complement Activation-Related Pathophysiological Changes in Anesthetized Rats: Activator-Dependent Variations of Symptoms and Mediators of Pseudoallergy. Molecules. 2019;24(18):3283. Published 2019 Sep 9. doi:10.3390/molecules24183283
7. Őrfi E, Mészáros T, Hennies M, et al. Acute physiological changes caused by complement activators and amphotericin B-containing liposomes in mice. Int J Nanomedicine. 2019;14:1563-1573. Published 2019 Feb 26. doi:10.2147/IJN.S187139
8. Parente R, Clark SJ, Inforzato A, Day AJ. Complement factor H in host defense and immune evasion. Cell Mol Life Sci. 2017;74(9):1605-1624. doi:10.1007/s00018-016-2418-4
9. Hong D, Sloane DE. Hypersensitivity to monoclonal antibodies used for cancer and inflammatory or connective tissue diseases. Ann Allergy Asthma Immunol. 2019;123(1):35-41. doi:10.1016/j.anai.2019.04.015
10. Szebeni J, Muggia F, Barenholz Y. Case study: Complement activation related hypersensitivity reactions to PEGylated liposomal doxorubicin: Experimental and clinical evidence, mechanisms and approaches to Inhibition. In: Dobrovolskaia MA, McNeil SE, editors. Handbook of Immunological Properties of Engineered Nanomaterials, 2nd Edition: World Scientific Publishing Company; 2015. p. 331-61.
11. Mészáros, T.; Csincsi, Á.I.; Uzonyi, B.; Hebecker, M.; Fülöp, T.G.; Erdei, A.; Szebeni, J.; Józsi, M. Factor H inhibits complement activation induced by liposomal and micellar drugs and the therapeutic antibody rituximab in vitro. Nanomedicine 2016, 12, 1023–1031.

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Appendices

List of publications, Curriculum vitae, Acknowledgements

Publications from this thesis

Fülöp T.; Kozma G.T., Vashegyi I., Mészáros T., Rosivall L., Urbanics R., Storm G., Metselar J.M., Szebeni J. Liposome-induced hypersensitivity reactions: risk reduction by design of safe infusion protocols in pigs. Journal of controlled release. 309:333-338
doi: 10.1016/j.jconrel.2019.07.005. (2019)

Fülöp T.; G., Nemes R., Mészáros T., Urbanics R., Kok R.J., Jackman J. A., Szebeni J. Complement activation in vitro and reactogenicity of low-molecular weight dextran-coated SPIONs in the pig CARPA model: Correlation with physicochemical features and clinical information. Journal of controlled release, 270:268-274. DOI: 10.1016/j.jconrel.2017.11.043 (2018).

Fülöp T.; Metselaar J.M., Storm G., Szebeni J. The role of thromboxane A2 in complement activation-related pseudoallergy. European Journal of Nanomedicine. 9. 10.1515/ejnm-2016-0039 DOI: 10.1515/ejnm-2016-0039 (2017).

Fülöp T., Józsi M., Metselaar J.M., Storm G., Rosivall L., Szebeni J. The possible role of factor H in complement activation-related pseudoallergy (CARPA): A failed attempt to correlate blood levels of FH with liposome-induced hypersensitivity reactions in patients with autoimmune disease. European Journal of Nanomedicine 7:7-14, DOI:10.1515/ejnm-2015-0004 (2015).

Fülöp T.; Mészáros T., Kozma G.T., Szebeni J., Józsi M. Infusion Reactions Associated with the Medical Application of Monoclonal Antibodies: The Role of Complement Activation and Possibility of Inhibition by Factor H. Antibodies, 7, 14 DOI: 10.3390/antib7010014 (2018).

Curriculum Vitae

Gyula Tamás Fülöp was born on 01 February 1990, in Kaposvár, Hungary. After finishing his Gymnasium in Kaposvár in 2008, he started his bachelor degree in Krems and der Donau, Austria at IMC University of Applied Sciences Krems. In 2011 he had a bachelor training semester in Amsterdam, the Netherlands at AMC Amsterdam investigating cancer stem cells. He got his bachelor degree in medical and pharmaceutical biotechnology, and right away started his master in the same field at IMC University Krems. He had his master training semester in Madrid at Centro de Investigaciones Biologicas, in the field of TGF-beta and endothelial cell research. He received his master diploma in 2013. He started his PhD studies at Semmelweis Medical University in Budapest, Hungary, at the Nanomedicine Research and Education Center supervised by Dr János Szebeni, Dr Gert Storm, Dr László Rosivall and Dr Josbert Metselaar. The PhD project was in the field of nanomedicine safety, focusing on hypersensitivity reaction prediction and prevention in in vitro and in vivo models. The PhD project was initiated with a collaboration between University of Twente and Semmelweis University, leading to a PhD thesis defence at the University of Twente.

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