

## Review

# Hemocompatibility testing for nanomedicines and biologicals: predictive assays for complement mediated infusion reactions

János Szebeni\*

Nanomedicine Research and Education Center, Semmelweis University and Bay Zoltán Applied Research Nonprofit Ltd., Budapest, Hungary

## Abstract

Infusion, or hypersensitivity reactions (HSRs) are frequent side effects of i.v. administered nanomedicines and biologicals. These, mostly mild and well tolerated, but occasionally severe or fatal allergic reactions represent a hemo-incompatibility due to activation of the complement (C) system. This review details the HSRs caused by marketed nanomedicines (liposomal drugs, micellar systems, polymer-conjugates of proteins, imaging agents, drug carrier nanosystems) and antibody therapeutics (mAbs), pointing out the remarkable similarity of clinical symptoms and difference from true (IgE-mediated) allergy. Beside the essentials, such as terminology, prevalence, risk factors and molecular and cellular mechanism of C activation-related pseudoallergy (CARPA) caused by the above agents, the paper highlights the biological rationale of these reactions, i.e., misinterpretation of nanoparticulate drugs by the immune system as pathogenic viruses, and C activation being an inherent function of mAbs. The public, as well as regulatory agencies are increasingly aware of the safety risks of possibly severe adverse immune reaction of drugs in the above categories, expressing need for new and appropriate immune toxicity tests in the preclinical stage. Here we outline and comment on the available methods for C activation and CARPA testing in vitro and in vivo, namely, the ELISA of C cleavage products (C3a, C5a, C4d, Bb, SC5b-9), the hemolytic (CH50) C assay, FACS measurement of basophil leukocyte activation, a – potentially – multiplex bead assay for C activation byproducts, the porcine assay of nanoparticle-induced cardiopulmonary distress and other CARPA tests in animals. The proven parallelisms between C activation and HSRs in vivo provide rationale for using these tests as predictors of infusion hypersensitivity, and the review suggests a decision tree for their use.

**Keywords:** adverse drug reactions; allergy; antibody; hypersensitivity; liposome; nanoparticles.

## Contents

1. Hemocompatibility assays: available tests and shortcomings .....	33
2. Hemocompatibility problems with nanomedicines and biologicals .....	34
3. Hypersensitivity and its risk factors .....	35
4. An adverse role of complement: mediation of allergy ...	35
5. The CARPA concept and terminology .....	35
6. Causes of recognition of nanomedicines and biologicals by complement .....	36
7. Mechanism of CARPA .....	38
8. Prevalence of CARPA .....	39
9. CARPAgenic drugs and agents .....	39
10. Specifics of CARPA caused by different drugs and agents .....	39
Liposome-induced CARPA .....	39
CARPA caused by monoclonal antibody therapeutics ...	39
11. Regulatory “reactions” to pseudoallergy as a safety issue .....	40
12. Scientific and clinical significance of CARPA .....	40
13. Predictive tests for CARPA .....	40
In vitro tests .....	40
ELISA assay of complement activation in normal human serum .....	40
ELISA assay of complement activation in whole blood .....	42
Examples for the use of complement ELISAs .....	42
Hemolytic assay of complement activation in animal blood or sera .....	45
FACS assay of SC5b-9 levels in human sera or plasma using cytometric bead array .....	46
FACS assay of basophil leukocyte activation in whole blood .....	47
The Porcine CARPA assay .....	50
Other animal models of CARPA .....	51
14. Decision tree on the use of CARPA assays .....	51
15. Summary .....	51
Acknowledgments .....	51
References .....	52

## 1. Hemocompatibility assays: available tests and shortcomings

Hemocompatibility testing, a major part of biocompatibility testing is the evaluation of critical interactions of foreign material *with blood* to explore possible adverse effects arising from the exposure of the foreign material to blood cells and proteins. Because such adverse effects are frequent and may represent serious health risks, introduction of blood-exposed materials in clinical use is highly regulated. Thus, official

\* Corresponding author: János Szebeni, MD, PhD, DSc, Nanomedicine Research and Education Center, Semmelweis University, Budapest, 1089 Nagyvárad tér 4, Hungary  
E-mail: jszebeni2@gmail.com

guidances by the USA Food and Drug Administration (FDA), European Medicines Agency (EMA) and other regulatory agencies are available, giving recommendations on assays that need to be conducted by the manufacturers to assure the lack of harm caused by blood-exposed foreign material.

Foreign material exposed to blood may fall into the categories of medical device, medicine and diagnostic agent. As for the hemocompatibility testing of medical devices, such as endovascular grafts, shunts, rings, patches, heart valves, balloon pumps, stents, pacemakers, hemopheresis filters, ISO 10993-4 (issued in 2002 and updated in 2009 by the International Organization for Standardization, the American National Standards Institute and the Association for the Advancement of Medical Instrumentation) (1) provides a list of recommended assays (Table 1). This list includes the testing of complement (C) activation, as the above devices often expose large surfaces to blood which provide surface for C deposition and, hence, C activation. As shown in the table, there are numerous assays available for testing C activation by such devices (2), partly replacing earlier regulated procedures in order to better discern alternative pathway (3) and whole cascade C activations (4).

As for the C tests evaluating the hemocompatibility of i.v. medicines and diagnostic agents, the same assays can be applied as recommended for medical devices, with appropriate adaptation for dispersed, water soluble or insoluble molecules. However, there is one particular form of i.v., drug-induced C incompatibility; infusion hypersensitivity, or infusion reactions, whose triggering is an inherent property of two relatively new classes of state-of-art therapeutic and diagnostic pharmaceuticals; nanomedicines and biologicals. Detailed guidance on the assessment of C-mediated infusion hypersensitivity to these drugs has not been included in any available regulatory documents, mainly because knowledge and experience in this uncharted intersection of cutting-edge pharmacology, non-standard toxicology and molecular immunology are relatively limited.

The goal of this review was to take an initiative along this line and outline the theoretical foundation of using C assays for the diagnosis and prediction of infusion hypersensitivity to nanomedicines and biologicals. In addition to the immunological aspects discussed, the review provides details on assay rationale, procedures and utility for diagnosis and prediction.

## 2. Hemocompatibility problems with nanomedicines and biologicals

Nanomedicines include a wide variety of synthetic and semi-synthetic drugs, agents and drug carrier systems whose complexity and size in the nanometer (nm) range distinguishes them from the traditional (Lipinski-type), low molecular weight medicines (5). Biologicals are also large molecular weight medicines distinguished by the source of their manufacture; living organisms and their products. The size range (8–20 nm) and molecular complexity of the best known biologicals, i.e., antibodies, serum proteins and enzymes would also qualify them as nanomedicines, however, for practical purposes, only functionally modified (e.g., pegylated or conjugated) biologicals are considered as nanomedicines.

A common feature of nanomedicines and biologicals is that while they are in the frontline of modern pharmacotherapy and have unprecedented market growth, they also have a unique toxicity problem that has not been solved to date: they can stimulate the immune system, leading to an allergy-like syndrome called hypersensitivity or infusion reaction. It is a major and potentially lethal hemo-incompatibility whose symptoms involve almost all organ systems (Table 2).

Based on its closer immune mechanism, infusion reactions can represent “real” allergy, one that arises after prior exposure of the reactogenic drug to blood and involves immune memory in the form of specific IgE formation. The other type involves no IgE and may arise, at least in part, as a consequence

**Table 1** Standard hemocompatibility assays.

Hemo-incompatibility	Assays
Thrombosis	Percent occlusion, flow reduction, pressure drop across device, antibody binding to thrombus components, thrombus mass, light microscopy and SEM of adhered platelets, leukocytes, aggregates, erythrocytes, fibrin, etc.
Coagulation	Coagulation PTT (non-activated), thrombin generation, specific coagulation factor assays, FPA, D-dimer, F1+2, TAT
Platelet activation	Platelet count/adhesion, platelet aggregation, template bleeding time, platelet function analysis, PF-4, thromboxane B2, platelet activation markers, platelet microparticles, gamma imaging of radiolabelled platelets, 111 In-labelled platelet survival
Blood cell changes	Leukocyte count with or without differential, leukocyte activation, haemolysis, reticulocyte count
Complement activation	C3a, C 5a, Bb, iC3b, C4d, SC5b-9, CH <sub>50</sub> , C3 convertase, C5 convertase

**Table 2** Symptoms of infusion-induced hypersensitivity reactions.

Cardiovascular	Broncho-pulmonary	Gastro-intestinal	Muco-cutaneous	Neuro-psychosomatic	Vegetative
Angioedema	Apnea	Nausea	Cyanosis	Back pain	Chills
Arrhythmia	Bronchospasm	Vomiting	Erythema	Chest pain	Diaphoresis
Cardiogenic shock	Coughing	Metallic taste	Flushing	Chest tightness	Fever
Hypertension	Dyspnea	Diarrhea	Rash	Headache	Sweating
Hypotension	Hyper-ventilation	Cramping	Rhinitis	Feeling of imminent death	Wheezing
Hypoxia	Laryngospasm	Bloating	Swelling	Fright	Rigors
Myocardial infarction	Pulmonary infiltrates		Urticaria	Panic	Feeling of warmth
Tachycardia	Respiratory distress		Nasal congestion	Rigors	
Ventricular fibrillation	Shortness of breath		Wheezing	Tightness in chest, throat	
Edema	Sneezing		Pruritis	Anxiety	
Death	Hoarseness		Angioedema	Confusion	
Loss of consciousness	Pulmonary infiltrates		Tearing	Dizziness	
Syncope	Stridor		Conjunctival erythema		

of activation of the complement (C) system. Hence its name: C activation-related pseudoallergy (CARPA).

Complement activation may be a contributing mechanism to infusion reactions, or can be its sole cause, and has numerous unique consequences relative to IgE-mediated allergy as outlined in Table 3. Most importantly, true allergic infusion reactions are observed only after repeated exposure of the reactogenic drug to blood and they get stronger upon repeated administration, while CARPA develops at the first exposure and the reaction loses strength with time and repetition. Table 3 also shows the scoring of infusion reactions arising from true allergy or CARPA (6).

### 3. Hypersensitivity and its risk factors

The terms “hypersensitivity reaction” or “allergic reaction” have broad and narrow meanings. The broad meaning originates from the classical categorization of these reactions by Gell and Coombs (7, 8) to four types (I-IV), which essentially cover all abnormal immune phenomena in human diseases. In contrast, the everyday meaning of hypersensitivity or allergy is an acute immune reaction to an allergen, including pollens, chemicals, drugs and a variety of other agents. The state of hypersensitivity to a drug or agent, i.e., proneness to develop true or pseudoallergy, have inherited as well as acquired contributing factors. Table 4 lists the best known risk factors for both true and pseudoallergy.

### 4. An adverse role of complement: mediation of allergy

The C system is one of the four proteolytic cascades (beside the coagulation, fibrinolytic and kinin-callikrein systems)

in blood. It consists of some 35 glycoproteins, 13 of which are bound to cell membranes and 22 are soluble in plasma, yielding up to some 14 defined split products upon activation via three different pathways referred to as classical, alternative and lectin. As part of the humoral, non-specific arm of the immune system, C provides the frontline of immune defense, with capability to execute as well as organize the elimination of all types of foreign microbes, e.g., bacteria, viruses and yeasts. Complement activation is known to contribute to inflammation, specific immune response and immune clearance, and it also has other basic physiological functions in the body, such as tissue growth, regeneration and pregnancy. Complement over- or under-activity, or other malfunction of the system is a major underlying cause or co-factor in numerous diseases (10), including allergy and pseudoallergy. Further information on the system, in particular the specific protein changes in different illnesses and hemo-incompatibilities, along with their specific tests, are described in detail in a previous Clinical and Technical Review by *TECOmedical* (11).

A simplified scheme of the C cascade and its effector functions are shown in Figure 1.

### 5. The CARPA concept and terminology

The concept that C activation may underlie non-IgE-mediated infusion reactions to i.v. liposomes was proposed in 1999, when evidence was obtained that the hemodynamic changes in pigs, that mimicked the cardiovascular changes of patients following the infusion of liposomal doxorubicin (Doxil), were due to C activation (12). Later studies provided a long list of evidence for the causal role of C activation,

**Table 3** Distinguishing features and grading of true and pseudoallergy.

Property	True allergy (IgE-mediated)	Pseudoallergy (C-mediated)
Reaction at first exposure	No	Yes
Latency period upon repeated exposures	Shortens	Prolongs
Strength of repeat reaction	Ascending	Descending
Self-induced tolerance (tachyphylaxis)	No	Possible
Efficacy of anti-inflammatory premedication	No	Yes
Acute pulmonary infiltration	No	Yes
<b>Grading by symptoms</b>		
Grade I	Symptoms transient and mild, infusion interruption not indicated, intervention not indicated	
Grade II	Requires infusion interruption and/or therapy, symptoms promptly respond to symptomatic treatment (e.g. antihistamines, NSAIDs, narcotics, i.v. fluids), prophylactic medication indicated for 24 h	
Grade III	Prolonged (i.e., not rapidly responsive to symptomatic medication and/or brief interruption of infusion), recurrence of symptoms following initial improvement, hospitalization indicated for other clinical sequelae (e.g., renal impairment, pulmonary infiltrates)	
Grade IV	Life-threatening anaphylaxis, pressor or ventilatory support indicated	
Grade V	Death	

among which the specific inhibition by C blockers, the mimicking of symptoms by the C activator zymosan and the correlation between C activation and reactivity in pigs are the most important (10).

These factors, taken together with the fact that the reaction develops within minutes after starting the infusion of liposomes at first administration, thus ruling out a role of IgE, led to the proposal of the term C activation-related pseudoallergy (CARPA). Considering that major hemodynamic, bronchopulmonary and skin changes in pigs, as well as the cardiac arrhythmias correspond to the symptoms of liposome-induced hypersensitivity syndrome in man (Table 2), the porcine CARPA model represents an authentic animal model of

the human *severe* hypersensitivity reaction to liposomes and other drugs.

In regards of terminology it should also be mentioned that the World Allergy Organization-recommended name for these infusion reactions also known as anaphylactoid, or idiosyncratic reactions, is “non-allergic hypersensitivity” (13). However, this nomenclature is questionable in light of the convention of using allergy and hypersensitivity as synonyms. As for the novelty and rationale of the term CARPA, C activation by liposomes has been known for almost half a century (14–17), and the fact that C activation contributes to allergic phenomena has also been known for a long time (18). It is tying together the two phenomena to increase awareness of a potential cause of infusion reactions that may make this term useful.

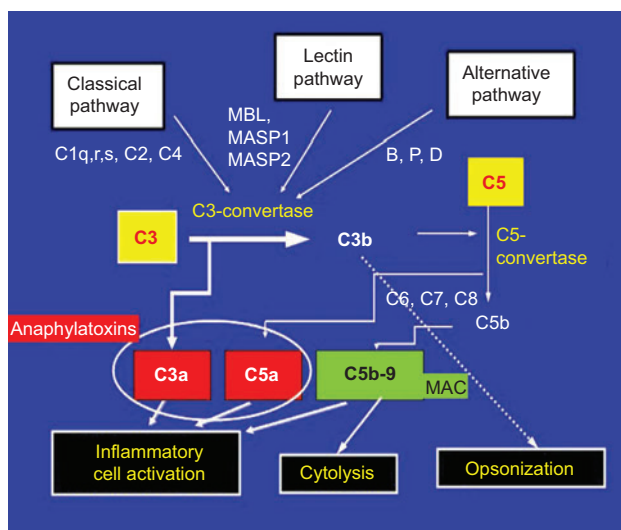
**Table 4** Risk factors for developing hypersensitivity reactions (9). Some risk factors for severe pseudoallergy.

Atopic constitution
Circulating lymphocyte counts $\geq 25,000 \text{ mm}^3$
Concomitant $\beta$ -adrenergic blocker therapy
Concurrent autoimmune disease
Female gender
History of drug allergy
Old age
Patients with lymphoid malignancies (mantle cell lymphoma, CLL)
Preexisting cardiac or pulmonary dysfunction

## 6. Causes of recognition of nanomedicines and biologicals by complement

In the case of non-proteinaceous nanomedicines, which consist of normally non-immunogenic molecules or polymers, it is their size (in the 50–200 nm range) and surface characteristics (molecular arrays or repetitive elements which are recognized by pattern recognition receptors on immune cells) which make them resemble human pathogenic viruses recognizable by the C system.

Figure 2 illustrates the sizes of drug carrier nanosystems, in relation to the “window of immune vision”, i.e., the size and molecular weight thresholds of cellular and humoral



**Figure 1** The Complement cascade and its effector functions.

immunity in recognizing particles as foreign. The figure suggests that liposomes and certain carbon nanotubes are within the spotlight of immune surveillance (blue triangle), while fullerenes, micelles, dendrimers, conjugated polymers, polymeric micelles or vesicles, aptamers, quantum dots, superparamagnetic iron oxide nanoparticles (SPIONs), polymeric micelles, nanocrystals all fall beyond immune recognition, at least in monomeric form. Consequently, the immune reactivity of these theoretically stealth particles probably involve interactions with blood elements or

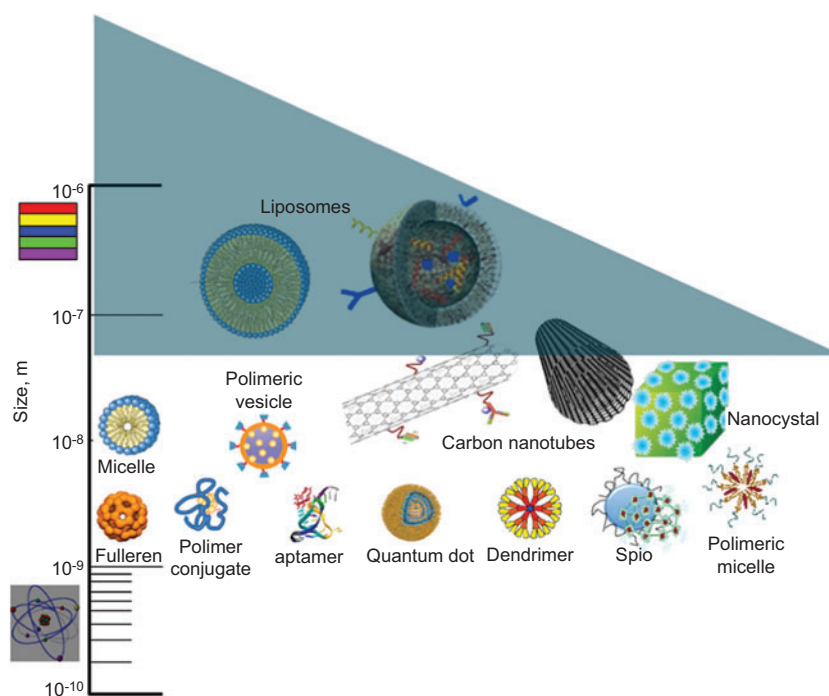
other effects which make them recognizable by the immune system.

Figure 3A, on the other hand, reminds that most pathogenic human virus classes in the 40–300 nm range look very much like small unilamellar (SUV) and multilamellar (MLV) liposomes, with Doxil, the first FDA-approved nanomedicine (19), is actually almost indistinguishable from HIV-1 (Figure 3B).

In addition to their similarity to viruses in terms of size, there is another major reason why nanomedicines are recognized by C as foreign; the absence of membrane proteins that protect cells from C attack (Figure 4), or surface camouflaging that viruses use for C evasion.

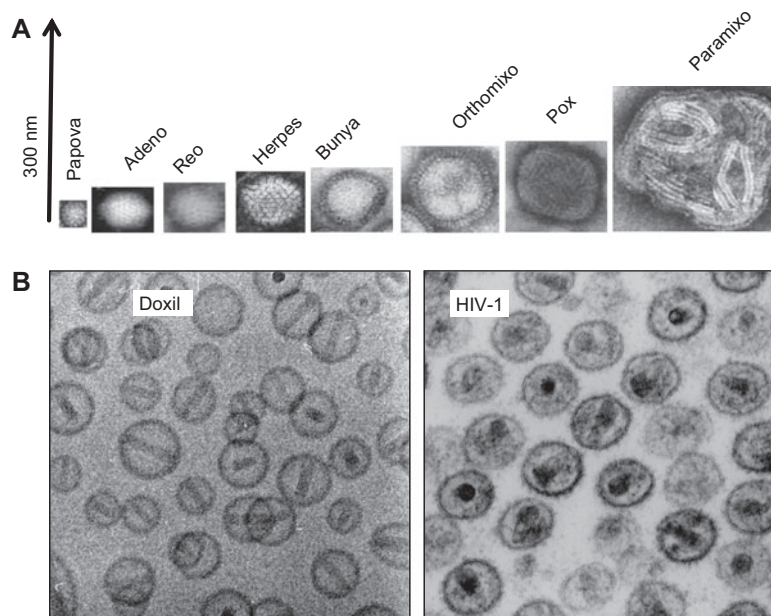
As for the cause of C recognition of biologicals, non-self proteins normally carry numerous antigenic epitopes to which the body responds with antibody production. Once such antibodies are formed, they bind to the foreign proteins and activate C, which is one of their roles in immune defense. Different antibody classes have different capability to activate C, with IgM being the most potent.

The C binding function of IgGs is well-known in immunology as well as in the industry of biologicals, leading developers to produce “C-stealth” humanized proteins (humanized antibodies) for intravenous use. Nevertheless, once they bind to their target, humanized antibodies also activate C, as C binding is an intrinsic function of certain IgG antibodies, regardless of their origin. This very basic principle is illustrated in Figure 5, reminding of the mechanism by which even totally humanized, immunologically fully matched monoclonal antibodies activate C if they bind to their target epitopes.

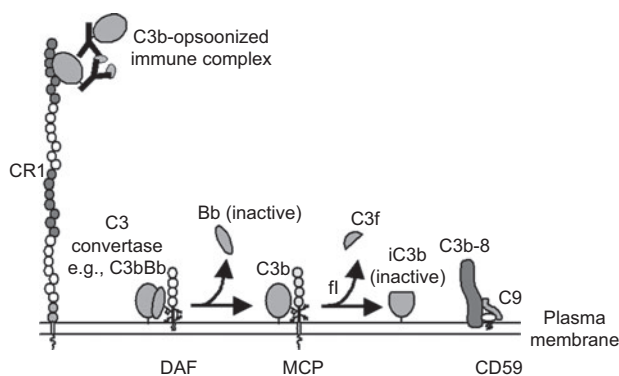


**Figure 2** Drug carrier nanosystems and their visibility by the immune system.





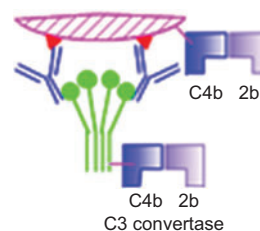
**Figure 3** Similarity between liposomes and pathogenic human viruses (A) and between Doxil and HIV (B).



**Figure 4** Membrane protein protecting host cells against C attack.

## 7. Mechanism of CARPA

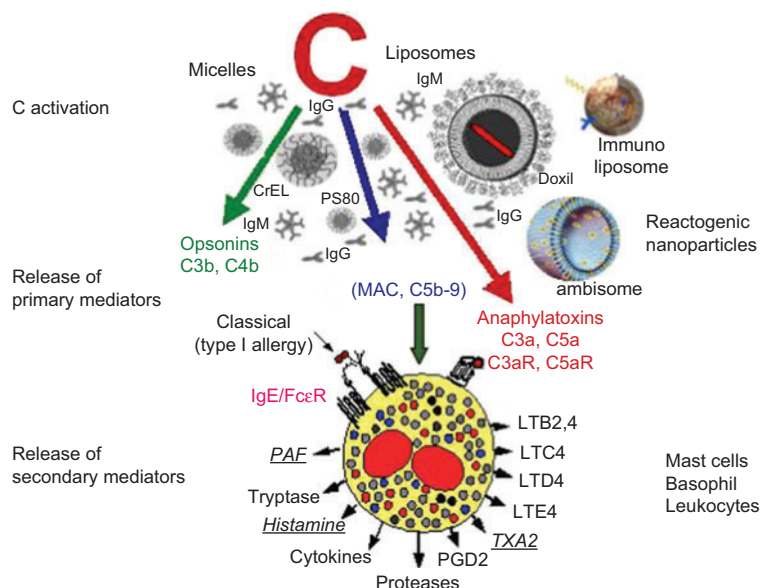
In keeping with the similarity of true allergic reactions and CARPA (Table 2), the immune cells responsible for allergy are also responsible for CARPA. These include mast cells, basophils and macrophages that express a group of G-protein coupled receptors which bind anaphylatoxins (i.e., C3a/C5a/C5L2 receptors). Binding of C activation byproducts, C3a and C5a to these receptors can trigger essentially the same intracellular signal cascade that is activated upon the engagement of allergen to membrane-bound IgE, leading to the release of a battery of secondary vasoactive mediators, including histamine, tryptase, PAF, leukotrienes (LTB<sub>2</sub>, LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, TXA<sub>2</sub>, PGD<sub>2</sub> and TXD<sub>4</sub>) (Figure 6). Some of these “allergomedins” (e.g., PAF, histamine, tryptase and TXA<sub>2</sub>) are preformed and liberate from the cells immediately upon activation, while others are de novo synthesized and, hence,



**Figure 5** Complement activation (C3 convertase formation) by surface bound IgG.

liberate slower. In the next step of CARPA allergomedins bind to their respective receptors on endothelial and smooth muscle cells, modifying their function in ways that lead to the symptoms of CARPA (Table 2).

It is notable in regards to the huge variety of CARPA symptoms that in addition to the above multistep, complex pathomechanism of mast cell activation, different individuals and tissues have different patterns of allergomedin receptors, and these receptors mediate different functions in different tissues. For example, skin and cardiac mast cells respond to different allergomedin stimuli (20). Furthermore, activation of H1 receptors leads to vasoconstriction and vascular leakage, and is responsible for the cardiovascular and cutaneous symptoms of anaphylaxis, while H2 receptors increase cellular cAMP levels and cause vasodilation, increased heart rate and pulse pressure (21). Increased vascular permeability, a hallmark sign of severe (Grade IV) CARPA may entail the transfer of up to 50% of intravascular fluid into the extravascular space within 10 min (22).



**Figure 6** Mechanism of CARPA.

## 8. Prevalence of CARPA

It has been estimated that as many as 30% of hospitalized patients may have a drug reaction of some type, with the incidence of severe and fatal reactions being approximately 7% and 0.3%, respectively (23). These statistics imply roughly 2 million serious reactions per year with ~100,000 fatalities, making adverse drug reactions the fourth to sixth leading cause of death in the USA (23). Another analysis pointed out that about 25% of all adverse drug reactions are of allergic nature (2) and about 77% of which is non-IgE-mediated, i.e., represent pseudoallergy (24). These statistics imply approximately ~400,000 severe and ~20,000 fatal pseudoallergic reactions each year in the USA.

## 9. CARPAgenic drugs and agents

There are a great number of nanomedicines reported to cause infusion reactions, with particular details incorporated in Tables 5–9 for liposomal-, micellar-, antibody based-, conjugated- and miscellaneous other drugs, respectively.

There are also some drug carrier systems whose C activating capabilities have been shown in vitro (e.g., poloxamers, carbon nanotubes) (25–31).

## 10. Specifics of CARPA caused by different drugs and agents

### Liposome-induced CARPA

Liposomes or other types of phospholipid assemblies are increasingly used in medicine for targeted or controlled release of various drugs and diagnostic agents. At present,

more than a dozen liposomal drugs are in the market, and more in advanced clinical trials. Table 5 lists those liposomal drugs in the market that have been reported to cause CARPA. The frequency of reactions reported to different drugs varies between 3% and 45% (32). Out of these, the reactions to Doxil have been studied in most detail, in humans as well as animals. Complement activation by Doxil, documented in several studies (33, 34), have been correlated with clinical symptoms in humans (35) and pigs (12, 26, 34). The main conclusions about C activation by Doxil and other liposomes are summarized in Table 10.

### CARPA caused by monoclonal antibody therapeutics

Most, if not all of more than 20 mAb-based pharmaceuticals approved to date carry a risk for causing infusion reactions. Table 7 provides specific information on the symptoms caused by these agents, which are essentially the same as listed for liposomes (Table 5), i.e., typical symptoms of infusion reactions (Table 2). However, there is a clear difference between mAb-induced and nanoparticle-induced CARPA, inasmuch as most mAb reaction starts somewhat later, mostly after 30 min, compared to immediate start of symptoms in the case of liposomes and micellar drugs. This difference can most easily be rationalized by the different kinetics of C activation in the case of nanoparticles and mAbs. Namely, while nanoparticles bind C almost immediately on their surface, mAbs need to undergo steric changes to become C activators (see Figure 5). It is widely known in molecular immunology that when antibodies bind to foreign surfaces, steric changes in the hinge region free up the C binding site on the Fc region. With mAb therapy, the relatively slow kinetics of target binding will most likely control the rate of C activation and other immune consequences of Ab binding.

**Table 5** Liposomal drugs causing infusion reactions.

Brand name (manufacturer)	Active ingredient	Indication	Type of particle (size)	Symptoms
Doxil, Caelyx (Johnson & Johnson)	Doxorubicin	Ovarian cancer, Kaposi sarcoma, myeloma multiplex	Liposomes (80-100 nm)	Flushing, shortness of breath, facial swelling, headache, chills, back pain, tightness in the chest or throat, hypotension
Myocet (Elan)				Flushing, dyspnea, fever, facial swelling, headache, back pain, chills, tightness in the chest and throat, hypotension
Abelcet (Elan, Enzon)	Amphotericin B	Fungal infections	Solid microparticles (1.6-11 $\mu$ m)	Shortness of breath, change in blood pressure
Ambisome (Gilead, Fujisawa)			Liposomes (45-80 nm)	Chills, rigors, fever, nausea, vomiting, cardiorespiratory events
Amphotec, Amphocyl (Elan)			Disk shape solid nanoparticles (115 nm)	Hypotension, tachycardia, bronchospasm, dyspnea, hypoxia, hyperventilation
DaunoXome (Gilead)	Daunorubicin	Kaposi sarcoma	Liposomes (45 nm)	Back pain, flushing, chest tightness
Visudyne (Novartis)	Verteporfin	Age-related macular degeneration	Multilamellar liposomes (multimicron)	Chest pain, syncope, sweating, dizziness, rash, dyspnea, flushing, changes in blood pressure and heart rate, back pain

## 11. Regulatory “reactions” to pseudoallergy as a safety issue

As shown in Table 1, C measurements are prescribed and widely applied for the hemocompatibility testing of medical devices, as regulated in ISO 10993-4 (1). However, C assays are not yet in the list of recommended tests by the regulatory agencies in relation to hypersensitivity or infusion reactions, although they can be lethal and it is possible to measure and predict these reactions as part of the safety evaluation of i.v. applied nanomedicines and biologicals. Nevertheless, the phenomenon is gaining increasing attention inasmuch as the “Immunotoxicity Evaluation of Biologicals” guideline by the US Federal Drug Administration (FDA) points to the possible need for predictive testing for C-mediated hypersensitivity reactions (37). Most recently, the European Medicines Agency’s guideline on the regulatory requirements for approval of a generic liposomal product requires “specific comment on hypersensitivity on infusion” (38).

## 12. Scientific and clinical significance of CARPA

Apart from better understanding the pathomechanism of HSRs, the CARPA concept offers a novel, functional categorization of HSRs that differentiates acute reactions (Type I) according to the underlying mechanism of mast cell and basophil release reactions. The scheme differentiates two major subclasses: (1) direct cell activation and (2) receptor-

mediated activation, with the latter category encompassing three subcategories: (a) IgE-triggered and FC receptor mediated, (b) anaphylatoxin-triggered and C3a/C5a receptor-mediated “CARPA” and (c) mixed type reactions, triggered by both IgE and anaphylatoxins (Figure 7).

The practical significance of the CARPA concept lies in the large number of these reactions, and their unpredictability. Besides the patients’ suffering and panic, it involves extra-care-related financial losses and, most importantly, it may deprive patients from the use of a potentially life-saving drug. The risk of death, although very low, may be an acceptable risk in the case of incurable cancer, but may be unacceptable in non-terminal illnesses.

## 13. Predictive tests for CARPA

### In vitro tests

#### ELISA assay of complement activation in normal human serum

**Goal** To quantify the C activating capability of test materials (liposomes, other nanoparticles, antibodies, etc) in individual or pooled normal human sera (NHS), taken as a measure of the risk for CARPA.

**Use** Preclinical safety assay assessing C-mediated immune reactivity of i.v. drugs and agents, as well as C-mediated individual hypersensitivity to them.



**Table 6** Marketed micellar drugs causing infusion reactions.

Brand name (manufacturer)	Active ingredient	Indication	Micelle-forming excipient (size)	Symptoms
Fasturec, Elitec (Sanofi Aventis)	Rasburicase	Hyperuricemia	Poloxamer-188 (~15 nm)	Anaphylaxis, bronchospasm, chest pain, diarrhea, dyspnea, fever, headache, hypotension, nausea, rash, rhinitis, urticaria, vomiting
Taxol (Bristol-Myers Squibb)	Paclitaxel	Cancer	Cremophor EL® (8-20 nm)	Acute respiratory distress, anaphylaxis, angioedema, arrhythmias, bronchospasm, chills, dyspnea, facial flushing, fever, flushing of the face and flushing of the upper thorax, hypertension, hypotension, rash, sudden death, tachycardia, urticaria, wheezing
Cyclosporine injection, USP (Draxis Pharma, Inc.)	Cyclosporine	Immuno-suppression		
Vumon injection (Bristol-Myers Squibb)	Teniposide	Leukemias		
Etoposide (Gensia Sicor Pharmaceuticals, Inc.)	Podophyllotoxin	Different cancers	Polysorbate 80 (8-16 nm)	Apnea, back pain, bronchospasm, chills, coughing, cyanosis, diaphoresis, dyspnea, fever, flushing, facial swelling, hyper and/or hypotension, laryngospasm, loss of consciousness, rash, tachycardia, tightness in throat, tongue swelling, urticaria
Taxotere (Sanofi-Aventis)	Docetaxel			Back pain, bronchospasm, chest tightness, chills, dyspnea, erythema, fatal anaphylaxis, fever, flushing, generalized rash, hypotension

**Background** Complement activation plays a causal role in CARPA either as sole, or as co-trigger. However, C activation is not a rate limiting step in CARPA.

**Principle** The assay consists of incubating the test material in freshly drawn or previously frozen (at -80°C) and freshly thawed, undiluted NHS for up to 60 min and measuring with ELISA the production of C split products as a function of incubation time at 37°C.

#### Procedure

- **Serum collection.** Peripheral blood is drawn from healthy volunteers, blood is allowed to clot at room temperature for 15 min and the serum is separated by centrifugation (3,500 g, 10 min, 4°C). The supernatant is aliquoted and stored at -80°C.
- **Plasma collection.** The same procedure as above, except that 10 IU/mL heparin is added to the freshly drawn blood and it is immediately centrifuged to collect plasma.
- **Controls:** PBS, zymosan, heat aggregated IgG.
- **Incubation.** Serum or plasma is incubated with the test material at 37°C for 15–60 min at a drug to serum volume ratio of 1:5, e.g., 5-fold diluted drug and 20% diluted serum/plasma. During incubation aliquots are taken at different

times (e.g., 0, 5, 10, 20, 30, 45 and 60 min) and diluted in the sample diluent to which 10 mM EDTA is added to stop the reaction.

- **ELISA assay of C cleavage products.** (The measurement of C3a, C5a, C4d, Bb, SC5b-9) is carried out as described by the manufacturer (Quidel USA/TECMedical, Switzerland).

**Notes** Studies to date suggest that the detection of C activation does not necessarily mean that CARPA will also develop in an individual. It is strong C activation, i.e., massive elevation of C split products (>5–10-fold elevation over background at peak) which seems to correlate with HSR (35).

**Advantages** Highly sensitive and specific assays that not only quantify C activation but also differentiate among the activation pathways. C4d and Bb measure activation via the classical and the alternative pathways, respectively, while SC5b-9 measures activation via the whole chain.

**Limitations** There is substantial inter-individual variation in C response to an activator. This necessitates the testing of multiple sera in order to evaluate the frequency of reactions and the extent of activation in reactive sera.

**Table 7** Marketed therapeutic antibodies causing infusion reactions.

Brand name (manufacturer)	mAb, type (targetantigen)	Indication	Incidence	Symptoms
<b>Anti-cancer use</b>				
Avastin (Genentech/Roche)	Bevacizumab, recombinant humanized IgG <sub>1</sub> (VEGF-A)	Combination chemotherapy of metastatic colon, lung, kidney cancer and glioblastoma	< 3%, Severe: 0.2%	Chest pain, diaphoresis, headache, hypertension, neurologic signs and symptoms, oxygen desaturation, oxygen desaturation, rigors, wheezing
Campath (Genzyme)	Alemtuzumab)–IH, recombinant, humanized IgG <sub>1</sub> κ (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
Erbix (Bristol-Myers Squibb, Eli Lilly)	Cetuximab, chimeric IgG <sub>1</sub> κ (human 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)	Trastuzumab, humanized IgG <sub>1</sub> κ (human 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
Mylotarg (Pfizer Inc./Wyeth Pharmaceuticals)	Gemtuzumab ozogamicin, recombinant humanized IgG <sub>1</sub> κ (CD33 on hematopoietic cells)	CD33 positive acute myeloid leukemia in first relapse	<8%	Acute respiratory distress syndrome, anaphylaxis, dyspnea, fatal anaphylaxis, hypotension, pulmonary edema
Vectibix (Amgen)	Panitumumab, recombinant humanized IgG <sub>2</sub> κ (human EGFR)	KRAS+ metastatic colorectal carcinoma	1-4%	Anaphylactic reaction, bronchospasm, chills, fever, hypotension
Rituxan (Genentech)	Rituximab, chimeric IgG <sub>1</sub> κ (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
<b>Anti-inflammatory use</b>				
Remicade (Janssen Biotech, Inc.)	Infliximab, chimeric IgG <sub>1</sub> κ (TNFα)	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)	Omalizumab, recombinant, humanized IgG <sub>4</sub> (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 1 year after beginning regularly administered

## ELISA assay of complement activation in whole blood

**Goal** To quantitate the C activating capability of test materials (liposomes, other nanoparticles, antibodies, etc) in fresh whole blood, taken as a measure of the risk of CARPA.

### Background

**Use** Preclinical safety assay assessing C-mediated immune reactivity of i.v. drugs and agents, as well as individual C-mediated hypersensitivity to them.

**Principle** Similar tests as described above for serum and plasma, except that blood cells are also present. This may modify the C reaction via different influences of the cells, including the presence of anaphylatoxin receptors and C inhibitor surface molecules (Figure 4) on white blood cells, platelets and erythrocytes.

**Procedure** Same as described for serum and plasma.

**Advantages** Because of the presence of blood cells, this test provides a closer model of the human blood than the serum or plasma tests.

**Limitations** Care should be taken on the use of anticoagulants, as they may have a major impact on C activation: EDTA may entirely inhibit it, citrate may partially inhibit it, while heparin inhibits or increases it in a dose-dependent fashion (40). The only anticoagulant that is known not to influence C action is lepirudin (hirudin) (40).

### Examples for the use of complement ELISAs

#### Complement activation by Doxil in vitro and in vivo. Correlation with infusion reactions in cancer patients

Figure 8 provides information on various aspects of C activation by Doxil in human serum in vitro: its frequency (A), dose dependence (B) and time course (C). Under the conditions of these studies (33) sera of seven out of 10 normal humans displayed sensitivity to Doxil, inasmuch as their SC5b-9 levels significantly increased over baseline within 10 min incubation at 37°C (bars with asterisks). The reaction reached its maximum at around 0.4 mg/mL Doxil (B), i.e., at 5-fold dilution of the content of the original vial in whole serum, and its kinetics reached plateau after about 15 min (33).

Figure 9 shows significant elevation of SC5b-9 over baseline in 21 out of 29 cancer patients at 10 min after infusion of Doxil for the first time, indicating C activation by the drug in

**Table 8** Marketed protein conjugates causing infusion reactions.

Brand name (manufacturer)	Conjugate	Indication	Symptoms
Adagen (Enzon)	PEG-adenosine deaminase	Immunodeficiency	Acute respiratory distress syndrome, anaphylaxis, angioedema, arthralgia, bronchospasm, chills, dyspnea, edema, erythema, fever, flushing, hives, hypotension, induration, injection site reactions, lip edema, pain, skin rash, swelling, tenderness, urticaria
Neulasta (Amgen)	PEG-Filgrastim (G-CSF)	Febrile neutropenia	
Oncaspar, Pegaspargase (Enzon, Rhône-Poulenc Rorer)	PEG-L-asparaginase	Lymphoblastic leukemia	

**Table 9** Miscellaneous other drugs causing infusion reactions.

Radio and ultrasound contrast agents	Enzymes Proteins Peptides	Miscellaneous other
Diatrizoate Iodixanol Iohexol Iopamidol Iopromide Iothalamate Ioversol Ioxaglate Ioxilan SonoVue Magnevist	Avonex Actimmune Abbokinase Aldurazyme Activase Zevalin Neupogen Neulasta Fasturtec Plenaxis	Cancidas Copaxone Orencia Eloxatin Salicylates

vivo (35) in the overwhelming majority of patients. Although C activation was observed in patients who showed no clinical HSR, the frequency of C reactors was significantly higher, and the rise of SC5b-9 more expressed in the reactor vs. non-reactor group, suggesting that in addition to the presence, the extent of C activation was also critical to the reactions. In fact, it is not merely the presence, but the extent of SC5b-9 rise that proved to correlate with HSRs, along with the rate of Doxil administration (35).

Figure 10 shows furthermore significant correlation between the infusion speed of Doxil, SC5b-9 elevation and the presence of HSR, which observation is consistent with the known reactogenicity of rapid infusion. Taken together, the above information provide validation for the use of SC5b-9 assay for predicting HSR with the provision that rather than the presence, it is the extent of C activation that correlates with CARPA (35).

#### Complement activation by micellar drug solvents

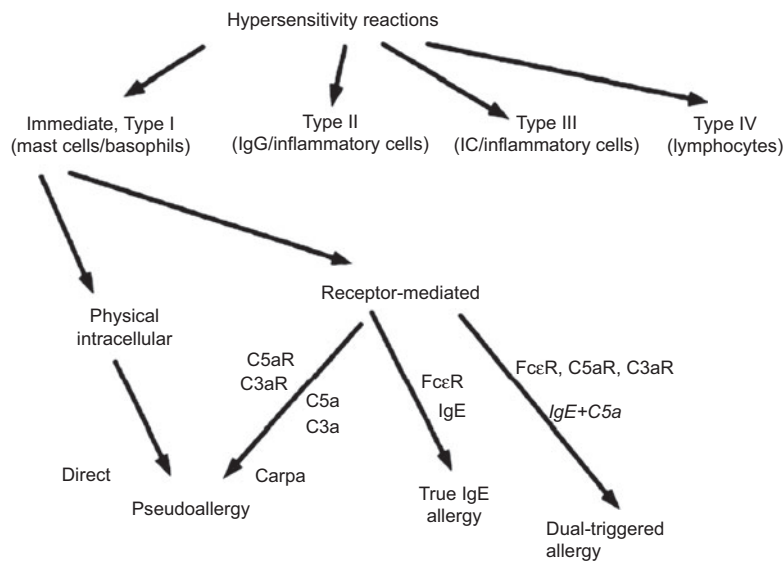
Figure 11 provides further validation of the use of SC5b-9 as a predictor of CARPA, inasmuch as it shows major C activation by both Cremophor EL and PS80 (Tween-80), which are known micellar vehicles of the active pharmaceutical ingredients (API) in Taxol and Taxotere, respectively, which are anticancer drugs that cause high rate of HSRs (Table 6) (39, 41, 42).

#### Comparison of serum and whole blood C assays

Figure 12 illustrates the differences among plasma, serum and whole blood in their SC5b-9 response to micelle-forming emulsifiers. Most remarkably, the baseline in serum is significantly greater than that in plasma and whole blood, suggesting that coagulation causes C activation which then

**Table 10** Features of liposomal C activation.

- All types of liposomes can activate C in human serum or plasma
- Sensitivity for C activation by different liposomes shows substantial individual variation
- Individual sensitivity for C activation is liposome formulation specific
  - A serum or plasma displaying C activation by certain liposomes is not necessarily sensitive to other liposomes
- C activation may proceed on both the classical and the alternative pathways
- In addition to IgG, IgM and C3, direct binding of CRP and C1q to liposomes can also trigger C activation
- C activation by liposomes is enhanced by:
  - Positive or negative surface charge
  - Increasing the size of liposomes
  - Inhomogeneity
  - Endotoxin contamination
  - Presence of aggregates
  - Presence of doxorubicin or similar drugs in the extraliposome medium
  - High percentage (>>50%) of cholesterol in the membrane
  - PEGylation of liposomes via negatively charged phospholipid anchors (e.g., DSPE)
  - Polyamino-coating
- C activation can be inhibited by known C inhibitors



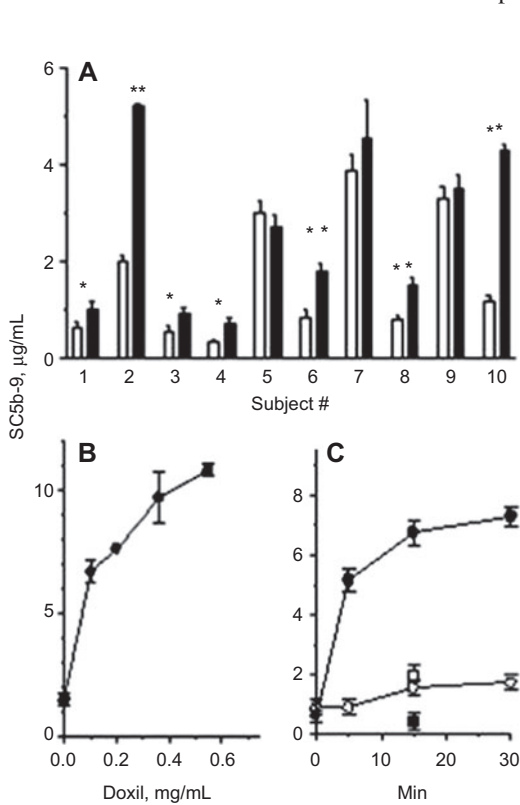
**Figure 7** Proposed new scheme of HSRs (25, 39).

influences the sensitivity of the specimen for extrinsic, micellar activators. Although the mechanism is not clear, this difference highlights the importance of using well-defined sources and reactants in C assays.

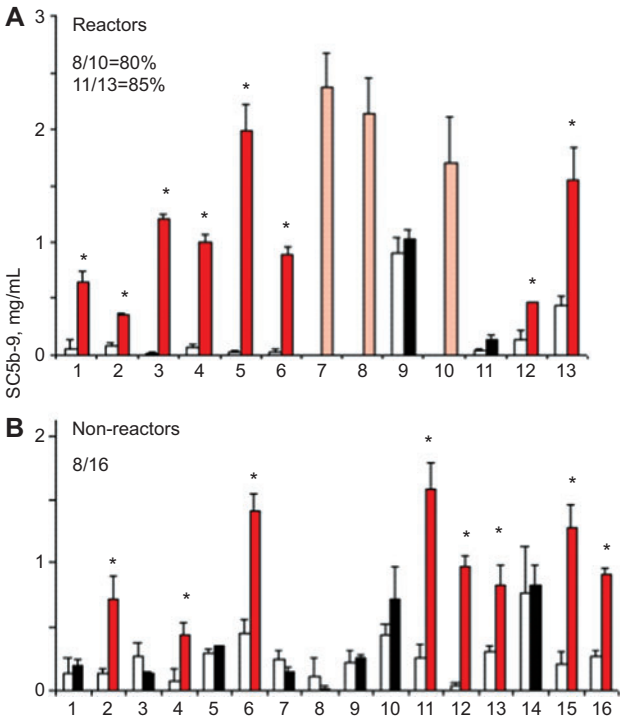
**Complement activation by monoclonal antibodies**

Figure 13A shows the effects of Erbitux and Herceptin on

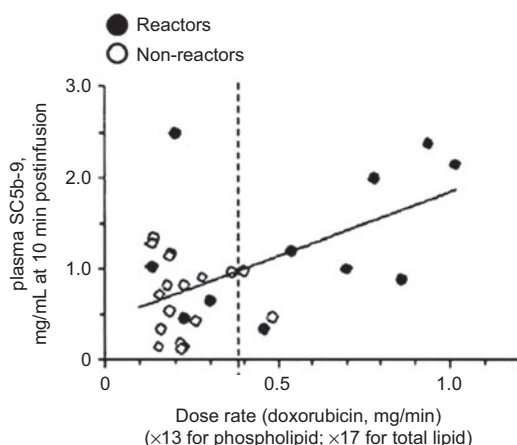
serum SC5b-9 in 20 NHS, suggesting no, or biologically minor activation (43). Likewise, measurement of SC5b-9 changes caused by in vitro incubation of cancer patients' sera with MabThera (Rituxan) gave relatively small elevation over baseline ( $21 \pm 0.18\%$ , mean  $\pm$  SE,  $n=21$ ) (unpublished



**Figure 8** Frequency, dose dependence and time course of C activation by Doxil.



**Figure 9** Baseline (empty) and maximal (at 10 min, filled bars) plasma levels of SC5b-9 in cancer patients displaying (A) and not displaying (B) infusion reactions following infusion of Doxil for the first time vivo. Red and rose bars significantly differ from baseline and average baseline, respectively (35).



**Figure 10** Correlations among Doxil dose rate ( $\times$ ), plasma SC5b-9 and the presence (filled circles) or absence (empty circles) of HSR in patients treated with Doxil for the first time (35).

observations). Nevertheless, preliminary data showed that combining all SC5b-9 rises obtained in sera of cancer patients incubated with MabThera ( $n=21$ ), Taxol ( $n=4$ ), Taxotere ( $n=1$ ) and Herceptin ( $n=4$ ), i.e., known reactogenic anticancer drugs, gave significant (Spearman's correlation:  $p=0.0143$ ,  $n=25$ ) correlation with the grade of HSRs they caused (Figure 13B).

Plotting the fraction of true positives out of the positives (TPR=true positive rate, sensitivity) vs. the fraction of false-

positives out of the negatives (FPR=false-positive rate) (Figure 13C), known as relative operating characteristic (ROC) analysis for diagnostic decision-making, also indicated significant ( $p=0.014$ ) predictive power for the SC5b-9 test. Nevertheless, it is almost certain that C activation by mAbs in human serum in vitro is not as effective as it is in the body upon binding of these antibodies to their target antigen.

### Hemolytic assay of complement activation in animal blood or sera

**Goal** To quantify the C activating capability of test materials (liposomes, other nanoparticles, antibodies, etc) in animal blood or serum, taken as a measure for the risk of CARPA.

**Background** The ELISA tests described above are specific for human C, they are incapable to measure C activation in animal blood. Rat and mouse C5a can be measured by specific ELISA kits sold, among others, by Antibodies-online Inc., Atlanta, GA), (Antibodies-online.com), while a porcine C5a ELISA is sold by Kamiya Biochem. Co. (Seattle, WA). Methods for porcine C5a have also been described in the literature (44). However, the use of C5a as a marker for in vivo C activation is problematic because of the rapid clearance of C5a from blood by C5aR-carrying cells (WBC, platelets, macrophages, etc), while specific ELISAs for the measurement of stable byproducts are not available. The classical C hemolytic (CH50) assay fills this gap as it measures total hemolytic activity in a species-independent manner.

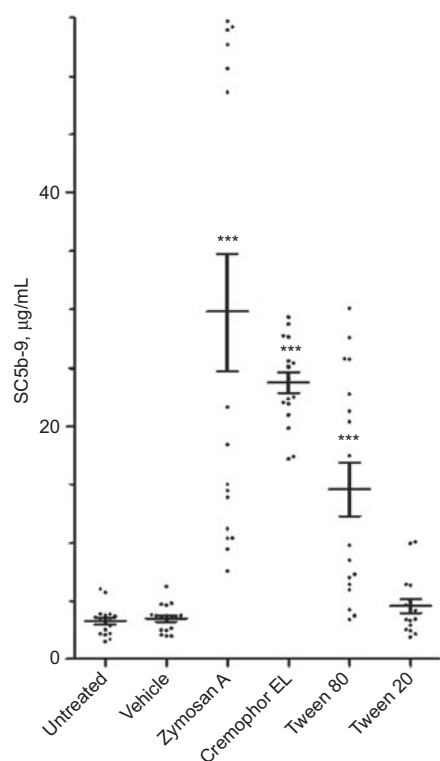
**Use** Preclinical safety assay assessing C-mediated immune reactogenicity of i.v. drugs and agents in animals, as well as individual C-mediated hypersensitivity to them.

**Principle** Mayer's total hemolytic capacity assay (CH50) was adapted to micro plate as follows.

### Procedure

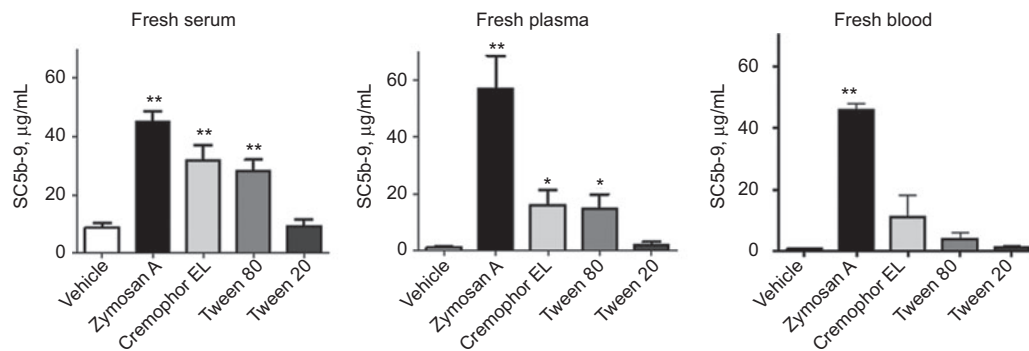
- Wash and suspend SRBC in GBV
- Measure total hemoglobin (Hb) content by lysing SRBC
- Prepare the erythrocyte suspension as calculated and mix thoroughly by vortexing
- Sensitize SRBC by adding rabbit anti-SRBC immunoglobulins (Hemolysin)
- Incubate with serum
- Pellet SRBC by centrifugation
- Measure Hb in supernatant at 540 nm
- Calculate specific hemolysis% and determine CH50%.

**Notes** The CH50 assay measures C consumption via “back measuring” the combined functional activity of all soluble C proteins that remains in serum after prior activation. It is

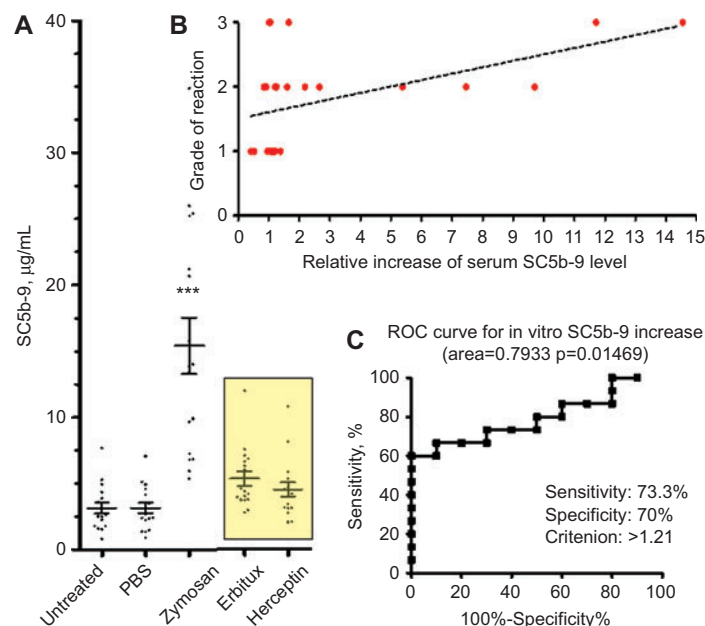


**Figure 11** Complement activation by micellar solvents used in highly reactogenic anticancer drugs Taxol and Taxotere.





**Figure 12** Comparison of C activation by different agents in serum, plasma and whole blood. (43).



**Figure 13** Complement activation by reactogenic mAbs and its correlation with CARPA in cancer patients.

considered as being less sensitive than most other ELISA assays of C cleavage products.

**Advantages** Species independent, inexpensive assay.

**Limitations** Relative low sensitivity. Due to the lability of some mouse C proteins, CH50 is not measurable in mouse serum.

**Examples** The CH50 assay was used previously to measure liposome-induced C activation in rat blood in vivo and in pig serum, in vitro. Table 11 shows the data from the latter study.

#### FACS assay of SC5b-9 levels in human sera or plasma using cytometric bead array

**Goal** To measure multiple C split products at the same time, using a particle-based, rapid assay.

**Use** Preclinical safety assay assessing the immune reactogenicity of i.v. drugs and agents, as well as individual hypersensitivity to them. Diagnostic test assessing C activation in various diseases.

**Background** Considering the wide range of pathogenic roles and diagnostic significance of C activation in human diseases, there has been significant interest in developing simple and fast, yet highly specific and sensitive assays for multiple C cleavage products at the same time.

#### Procedure

- Standards and samples are mixed and incubated with capture Ab-grafted beads at room temperature in the wells of 96-well plates.
- Beads are washed, suspended and incubated with FITC-conjugated detector antibodies at room temperature.

**Table 11** Complement consumption by various liposomes in pig serum. Data from (45).

Liposome	Complement consumption, % relative to baseline		
	1 mg/mL‡		8 mg/mL
	10 min§	30 min	5 min
DMPC/DMPG/Chol (50:5:45) LMV	18.9±4.2 (5)	32.5±7.6 (6)	59.6±14.7 (3)
DMPC (100) LMV	8.9±4.9† (5)	16.0±5.8† (6)	
DMPC/Chol (55:45) LMV	9.4±9.9* (6)	16.4±5.7† (6)	
DMPC/DMPG (95:5) LMV	13.9±7.2 (5)	24.7±8.7* (6)	
DMPC/DMPG/Chol (24:5:71) LMV	100±0† (3)		
DMPC/DMPG/Chol (50:5:45) LUV	-4.1±4.5† (5)	17.6±9.6† (6)	32.2±3.1† (3)

‡Final lipid concentration, §Incubation time. \*P, 0.05 or †P, 0.01.

- Beads are suspended, shaken analyzed for green (FITC) fluorescence in a flow cytometer or equivalent machine at 488 nm excitation.

### Advantages

- Enables multiplexing, i.e., simultaneous measurement of several C cleavage products.
- The procedure is shorter than conventional ELISA.
- With the use of state-of-art (miniaturized, or table) FACS machines, it can be applied as a bedside, rapid test particularly useful in bedside prediction of HSRs to i.v. drugs.

**Limitations** FACS is at present less accessible technology than ELISA.

**Example** Figure 14 shows the calibration curve of an SC5b-9 bead-based FACS assay, whose effective dynamic range (i.e., range of linearity) is between approximately 5 and 80 µg/mL, i.e., the range where this analyte changes in serum upon incubation with C activator liposomes, zymosan and other positive controls. Thus, it is not necessary to dilute the plasma or serum samples for the measurement of abnormal elevations of SC5b-9.

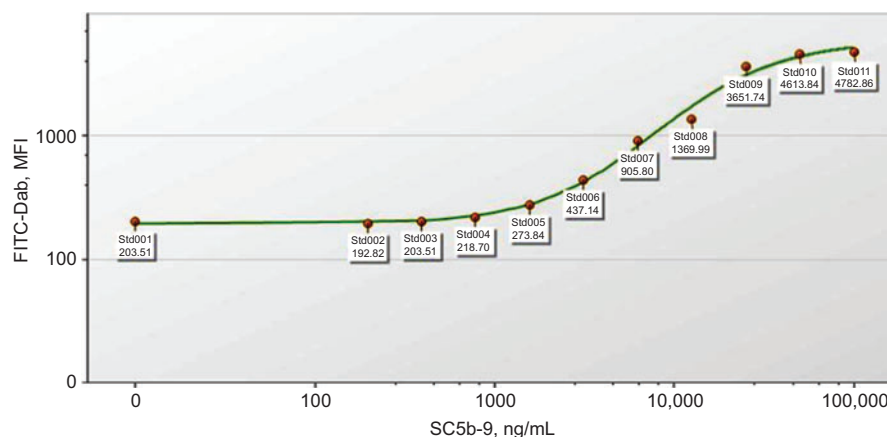
### FACS assay of basophil leukocyte activation in whole blood

**Goal** To quantitate the basophil leukocyte activating capability of i.v. drugs and agents (liposomes, other nanoparticles) in whole blood, as an alternative method to assess their risk to cause CARPA.

**Use** Preclinical safety assay utilizing Beckman-Coulter's Allergenicity Kit ([http://www.analis.com/files/VprodFiles/1609/BC\\_DS-Allergenicity%20kit.pdf](http://www.analis.com/files/VprodFiles/1609/BC_DS-Allergenicity%20kit.pdf)) to assess the basophil leukocyte response to i.v. drugs and agents, taken as an index of immune reactivity as well as individual hypersensitivity.

### Background

- Mast cells and their circulating form, basophil leukocytes, are first-line effector cells of allergic and pseudoallergic responses. Their activation via the IgE and/or anaphylatoxin receptors (C3aR, C5aR) and subsequent secretion of range of vasoactive allgomedins may play a crucial, rate limiting role in CARPA (Figure 6).
- The ectonucleosidase CD203c is a glycosylated surface molecule on mast cells, basophils and their precursors, whose upregulation has been used as a quantitative marker



**Figure 14** Calibration curve of the monoplex SC5b-9 CBA assay under evaluation (SeroScience Inc., in collaboration with Diatron Ltd., Hungary).

of the activation of these cells (46–48). CRTH2 is a constitutently expressed basophil cell marker, while CD3 is a lymphocyte marker.

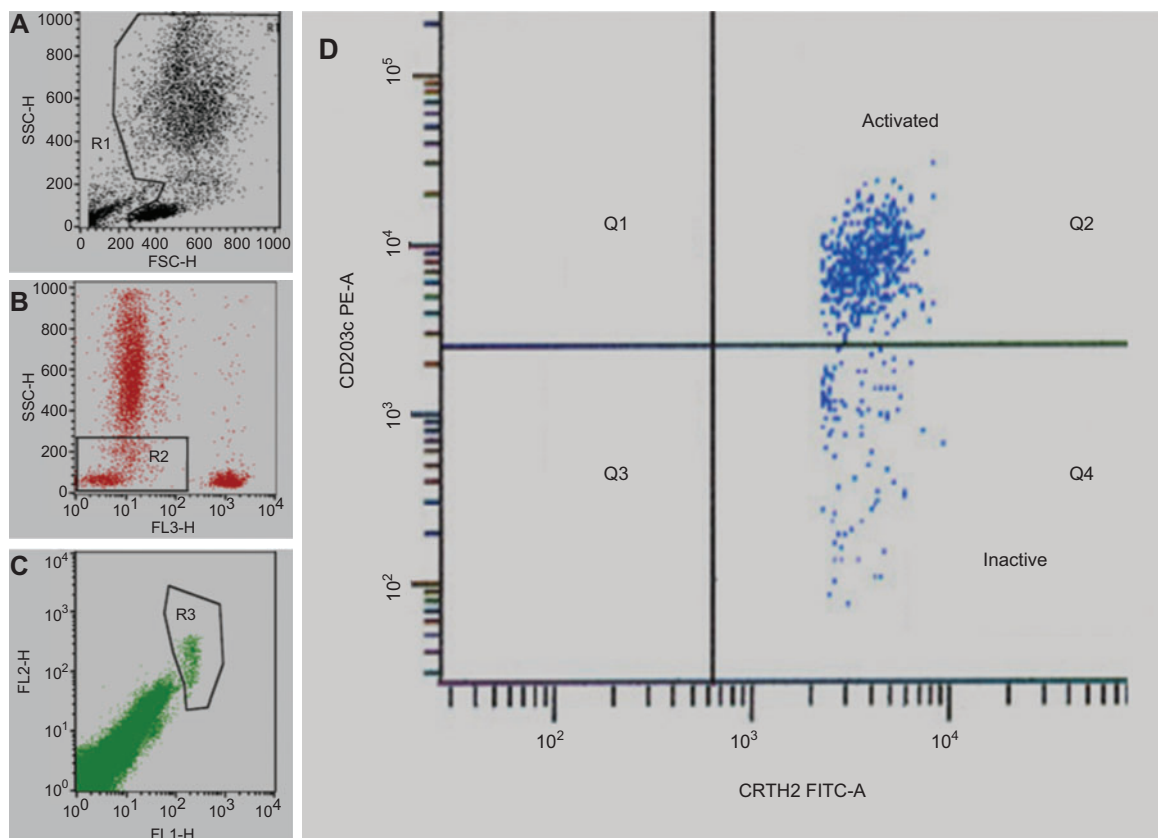
**Assay principle** Gating and counting of CD203c<sup>pos</sup>, CRTH2<sup>pos</sup> and CD3<sup>neg</sup> cells in a flow cytometer, after staining of WBC with antibodies specific against the above antigens and tagged with FITC, PE and PC7 fluorophores, respectively, enables quantification of basophil cell activation.

### Procedure

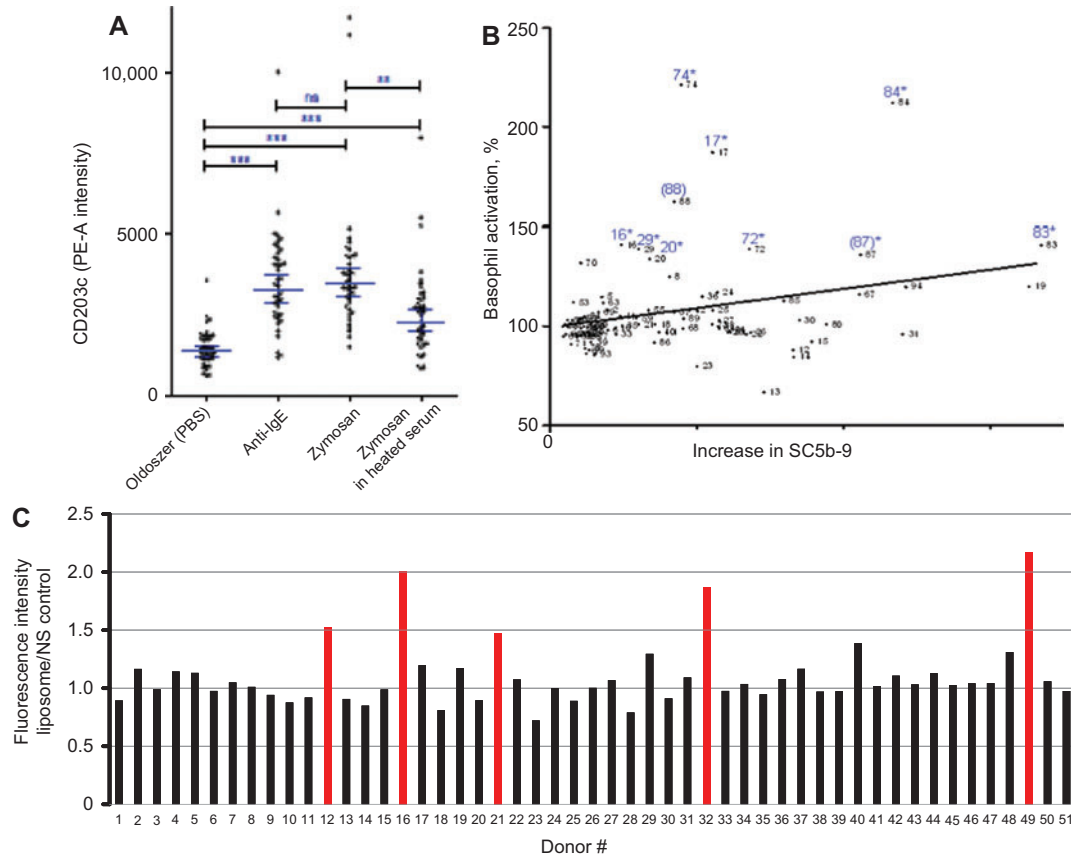
- **Blood collection.** Freshly drawn blood from healthy volunteers anticoagulated with 5 IU/mL heparin.
- **Incubation.** Collected blood is incubated with the test drugs and controls (i.e., rabbit anti-human IgE, zymosan) for 15 min at 37°C, protected from light.
- **Termination of the reaction.** Reaction is stopped with EDTA-containing PBS and the samples are treated with a “fix and lyse” solution provided in the kit that contains 0.1% formaldehyde and an undisclosed cyclic amine.
- **Staining of cells.** Fixed WBCs are incubated with the Ab mixture containing anti-CD203c-PE, anti-CRTH2-FITC and anti-CD3-PC7.

- **Acquire histograms.** After excluding CD3 positive cells (T lymphocytes) and gating on CRTH2<sup>pos</sup>CD203c<sup>bright</sup>CD3<sup>neg</sup> (Q2) and CRTH2<sup>pos</sup>CD203c<sup>dim</sup>CD3<sup>neg</sup> (Q4) (Figure 15A), basophils are counted (BC) in these regions and activation is expressed as the  $BC_{Q2} / BC_{Q2} + BC_{Q4} \times 100$ .
- A positive response is defined as a 10% increase in the number of cells expressing CD203c above the negative control in volunteers responding to the anti-IgE positive control.

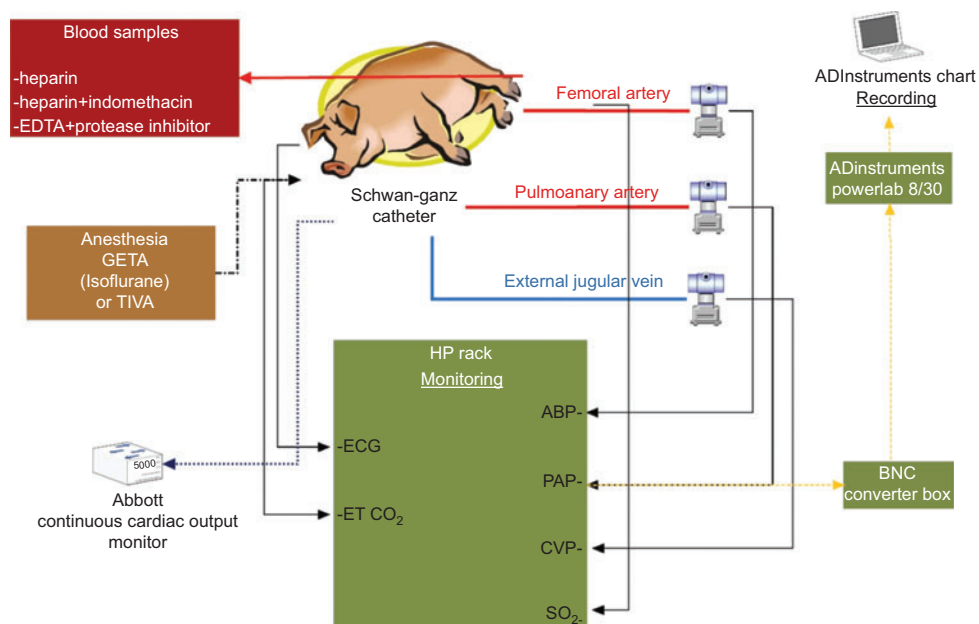
**Example** Figure 16A validates the assay for detecting C activation, inasmuch as it shows equal activation of basophils by anti-IgE and zymosan. That C activation contributes to the effect of zymosan is shown by the decreased activation in heated, and, hence, C-depleted serum. Figure 16B shows significant correlation between C activation in NHS and basophil activation in the corresponding whole blood samples ( $p=0.0058$ ). Figure 16C presents evidence that in five out of 51 normal human blood, liposomes caused >50% increase in CD203c fluorescence, indicating basophil activation in about the same percentage as that of liposome reactions in man. This observation suggests that mast cell activation may be the rate limiting step in liposome reactions, and that the basophil assay could be used to predict individual hypersensitivity to liposomal or other drugs.



**Figure 15** FACS assay of basophil activation. A, histogram displaying all events from which WBCs are delimited in region 1 (R1); B, histogram displaying R1, from which granulocytes and T cells are excluded (R2); C, histogram displaying R2, from which basophil cells are gated out on the basis of their CRTH2 positivity; D, Distinction of activated and non-activated basophils among CRTH2 positive cells based on their CD203c-PE fluorescence in a zymosan-treated sample.



**Figure 16** Assessment of the utility of the basophil activation assay in predicting CARPA. basophil assay Validation of the Beckman's FACS assay of basophil activation. A, histogram displaying all events from which WBCs are delimited in region 1 (R1); B, histogram displaying R1, from which granulocytes and T cells are excluded (R2); C, histogram displaying R2, from which basophil cells are gated out on the basis of their CRTH2 positivity; D, Distinction of activated and non-activated basophils among CRTH2 positive cells based on their CD203c-PE fluorescence in a zymosan-treated sample.



**Figure 17** Scheme of the porcine CARPA assay.

## The Porcine CARPA assay

**Goal** To predict the acute reactogenicity of selected nanoparticles in hypersensitive human individuals.

**Use** Preclinical safety screening assay (Figure 17).

**Background** Pigs are particularly sensitive for nanoparticle-induced cardiopulmonary distress and cutaneous changes, a feature that may be related to the presence of pulmonary intravascular macrophages (PIM cells) in this, as well as in other even-toed (hoofed) animals (Artiodactyla), including sheep and goats (49).

## Procedures

- Animals. Pigs (15–40 kg) of both sexes are sedated with intramuscular ketamine (500 mg) and anesthetized with 2% isoflurane, using an anesthesia machine, or with intravenous nembutal (30 mg/kg).
- A pulmonary artery catheter is advanced via the right internal jugular vein through the right atrium into the pulmonary artery to measure pulmonary artery pressure (PAP).
- A catheter is advanced in the femoral artery to measure systemic arterial pressure (SAP).

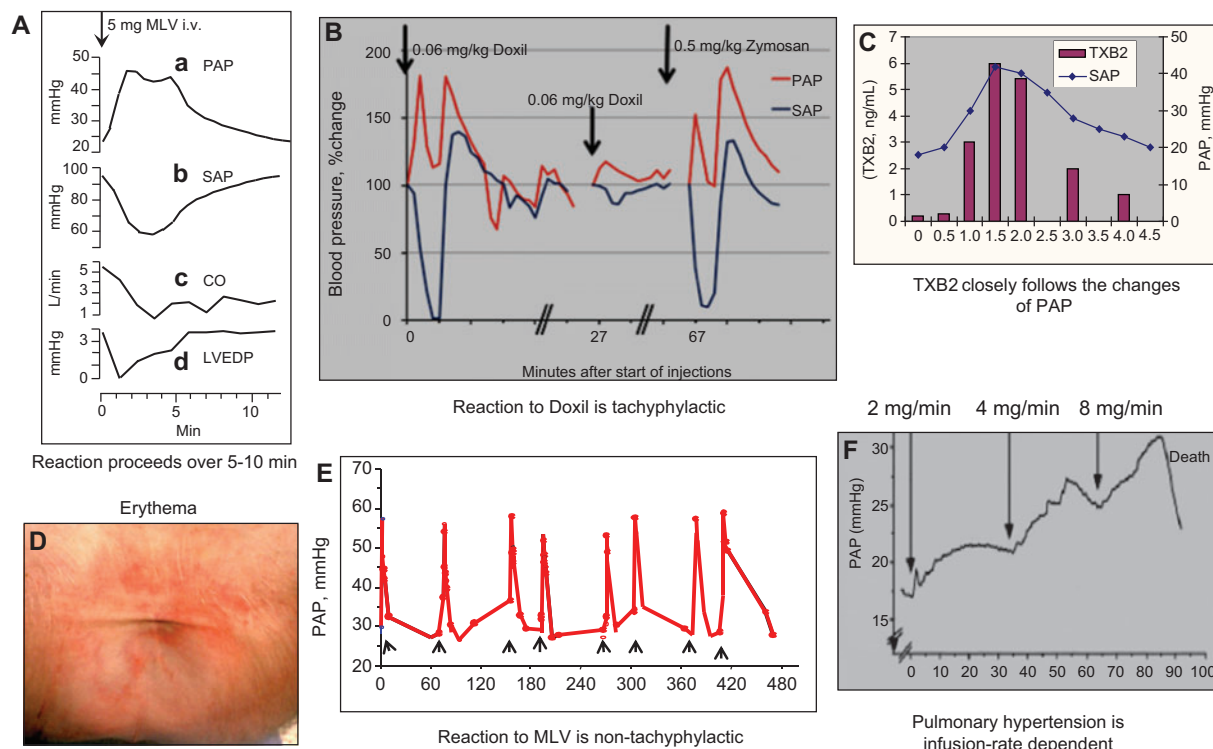
**Treatment** Liposomes or other test materials are diluted in phosphate buffered saline (PBS) and injected into the pulmonary artery as a bolus, via the pulmonary arterial catheter. The bolus is flushed into the circulation with 5–10 mL PBS.

## Endpoints

- CAS score: a semiquantitative measure of the severity of cardiac electric, circulatory (systemic and pulmonary), and skin changes during CARPA (26). Scores of 0–5 imply no response (CAS: 0), minimal (CAS: 1), mild (CAS: 2), moderate (CAS: 3), severe (CAS: 4), and lethal (CAS: 5) reactions. The symptoms specifying different CAS values are described in ref. (26).

## Notes

- The cardiopulmonary symptoms of pig CARPA mimics the most severe, grade IV or V allergic reactions in humans (Table 3) which involves hypotensive shock with cardiac arrhythmias, ventricular fibrillation and cardiac arrest, as well as the skin symptoms (flushing and rash). Thus, the model is appropriate for studying the mechanism and conditions of severe HSRs in hypersensitive man.
- The individual variation of hemodynamic reaction in pigs is relatively small.
- The pig CARPA model is uniquely capable to screen out immune reactive nanoparticles that may cause severe reactions in hypersensitive individuals.
- Although its oversensitivity implies false-positivity in terms of predicting reactions in normal humans, it needs to be remembered that infusion reactions cause real harm only in hypersensitive individuals, who are also not “normal” in their response to nanoparticles.



**Figure 18** Features of porcine CARPA pertinent to human liposome reactions.



**Table 12** Liposome-induced CARPA in different species: Dose dependence and symptoms. Up and down arrows indicate the rise or fall of the parameter specified, while the size of arrows is proportionate with the strength of response (28, 53–55).

Species	Trigger dose (mg PL/kg)	Manifestations						
		PAP	SAP	Skin	WBC	Platelets	TXB2	Histamine
Human*	0.01-0.2	↑	↑↓	+	↑↓	↓	?	↑
Pig	0.01-0.3	↑↑	↑↓	+	↑↓	↓	↑	?
Dog	0.05-0.1	↑	↓	-	↑↓	↓	↑	↑
Goat	0.1-0.2	?	↓	-	?	?	?	?
Rabbit	?	↑	↓	-	↓	↓	?	?
Rat	5-25	?	↓	-	↑↓	↓	↑	?
Mouse	?	?	?	-	↑↓	↓	?	?

**Examples** Over the past 13 years or so, we have injected numerous pigs and other animals (dogs and rats) with various liposomes and other nanoparticles (12, 26, 28, 34, 45, 50, 51).

Figure 18 illustrates some features of porcine CARPA that mimic the human liposome reactions and/or help in unveiling their mechanism or prevention. In particular,

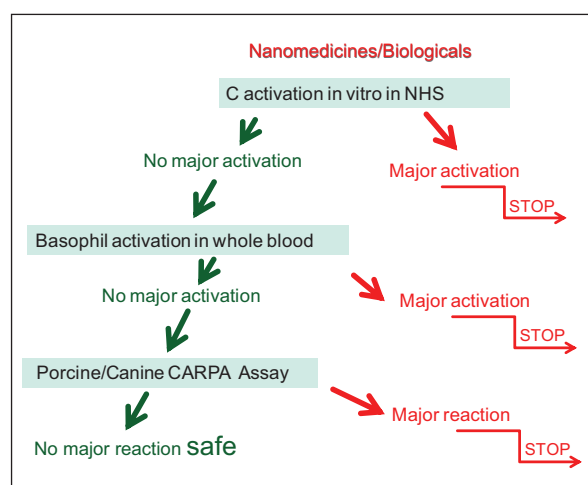
- the hemodynamic changes involve major rises in PAP, declines of SAP, cardiac output (CO) and left ventricular end-diastolic pressure (LVEDP) (33) (A);
- the reaction to Doxil is tachyphylactic, affording desensitization with empty (placebo) Doxil (Doxebo) (51) (B);
- the pulmonary reaction closely correlates with the rise of plasma thromboxane B2 (TXB2) (12) (C);
- the reaction can be associated with skin changes, such as flushing and rash (36, 52) (D);
- the reaction is non-tachyphylactic in the case of large multilamellar liposomes (12) (E);
- the reaction is infusion speed dependent (12) (F).

#### Other animal models of CARPA

CARPA can be induced by i.v. injection of liposomes and other nanoparticles in many other species, with sensitivity and symptoms differing from those seen in pigs (28). The sensitivity of to liposomal CARPA usually decreases in the following order: pig, dog, human (hypersensitive), rabbit, sheep, rat and mouse. The methods of detection and endpoints are similar or the same as described above, with obvious adaptations to the size differences among different animals. Table 12 tabulates some characteristic features of CARPA in pigs, dogs, goats, rats, rabbits and mice.

#### 14. Decision tree on the use of CARPA Assays

The flowchart in Figure 19 provides a scheme of possible sequence of assays assuring that a drug candidate nanomedicine or biological has minimal risk for CARPA in man.



**Figure 19** Scheme of possible sequence of CARPA assays for pre-clinical safety evaluation.

#### 15. Summary

The increasing use of nanomedicines and biologicals in pharmacotherapy and medical diagnosis give rising significance to CARPA, a generally mild and tolerable immune side effect whose medical significance lies in the risk that it might turn into an immune catastrophe in certain hypersensitive individuals. Predicting this event represents therefore an essential safety measure in these fields of drug R&D. The described information, principles, tests and decision tree may help in solving this issue. Further evaluation and refinement of the recommended assays will require more studies and expert feedback.

#### Acknowledgments

Thanks are due to all scientists and technicians who performed or contributed to the above-reviewed (published and unpublished) studies over many years, including Alving C, Baranyi, L, Barenholz C, Basta M, Báthori Gy, Bedőcs P, Benedek K, Bián

T, Bodó M, Bunger R, Fodor B, Hérincs Z, Kozma G, Mészáros T, Metselaar B, Milosevits G and J, Moghimi, M, Muggia F, Révész Cs, Robotka H, Rosivall L, Rozsnyay Z, Savay S, Storm G, Toth M, Urbanics R, Weiszhar Zs. Support for this review by Marieluise Wippermann, from TECOMedical group, Switzerland, is sincerely acknowledged.

## References

- Biological evaluation of medical devices— Part 4: selection of tests for interaction with blood, ANSI/AAMI/ISO 10993-4:2002/ (R) 10 March 2009, 2009.
- Harker LA, Ratner BD, Didisheim P. Cardiovascular biomaterials and biocompatibility. *Cardiovasc Pathol* 1993;2(Suppl):1S–224S.
- Standard Practice for Testing for Alternative Pathway Complement Activation in Serum by Solid Materials, ASTM, publication F2065-00, 1984.
- Standard Practice for Testing for Whole Complement Activation in Serum by Solid Materials, ASTM F1984-99, 1984.
- Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliver Rev* 1997;23:3–25.
- Lenz HJ. Management and preparedness for infusion and hypersensitivity reactions. *Oncologist* 2007;12:601–9.
- Gell PG, Coombs RR. Classification of allergic reactions underlying disease. Oxford: Blackwell, 1963.
- Coombs RR, Gell PG. Classification of allergic reactions responsible for drug hypersensitivity reactions. In: Coombs RA, Gell PG, editors. *Clinical Aspects of Immunology*, 2nd ed. Philadelphia, PA: Davis, 1968, p. 575–96.
- Vogel WH. Infusion reactions: diagnosis, assessment, and management. *Clin J Oncol Nurs* 2010;14:E10–21.
- Szebeni J. Complement activation-related pseudoallergy: mechanism of anaphylactoid reactions to drug carriers and radiocontrast agents. In: *The complement system: novel roles in health and disease*. Boston, MA: Kluwer, 2004:399–440.
- TECOMedical, Complement Diagnostics: Haemocompatibility testing of medical devices, pharmaceuticals and blood products, 2010.
- Szebeni J, Fontana JL, Wassef NM, Mongan PD, Morse DS, Dobbins DE, et al. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudo-allergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. *Circulation* 1999;99:2302–9.
- Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, et al. Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization. *J Allergy Clin Immunol* 2003;113: 832–6.
- Haxby JA, Kinsky CB, Kinsky SC. Immune response of a liposomal model membrane. *Proc Natl Acad Sci US* 1968;61:300–7.
- Haxby JA, Gotze O, Muller-Eberhard HJ, Kinsky SC. Release of trapped marker from liposomes by the action of purified complement components. *Proc Natl Acad Sci USA* 1969; 64:290–5.
- Kinsky SC, Haxby JA, Zopf DA, Alving CR, Kinsky CB. Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. *Biochemistry* 1969;8: 4149–58.
- Alving CR, Kinsky SC, Haxby JA, Kinsky CB. Antibody binding and complement fixation by a liposomal model membrane. *Biochemistry* 1969;8:1582–7.
- Dervadevics M, Hidvegi T, Schmidt B, Fust G, Varga L. Ragweed allergy: correlation between skin reactivity and in vitro complement activation. *Immunol Lett* 1998;64:119–23.
- Barenholz, Y. Doxil—the first FDA-approved nano-drug: from an idea to product. In: Peers D, editor. *Handbook of harnessing biomaterials in nanomedicine: preparation, toxicity, and applications*. Singapore: Pan Stanford Publishing Pte. Ltd., 2012: 335–98.
- Genovese A, Stellato C, Patella V, Lamparter-Schummert B, de Crescenzo G, Adt M, et al. Contrast media are incomplete secretagogues acting on human basophils and mast cells isolated from heart and lung, but not skin tissue. *Eur J Radiol* 1994;18(Suppl 1):S61–6.
- Lieberman P. The use of antihistamines in the prevention and treatment of anaphylaxis and anaphylactoid reactions. *Singapore Med J* 1989;30:290–3.
- Lieberman P. Anaphylactoid reactions to radiocontrast media. *Clin Rev Allergy* 1991;9:319–38.
- Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients. A meta-analysis of prospective studies. *J Am Med Assoc* 1998;279:1200–5.
- Demoly P, Kropf R, Bircher A, Pichler WJ. Drug hypersensitivity: questionnaire. EAACI interest group on drug hypersensitivity. *Allergy* 1999;54:999–1003.
- Szebeni J. Complement activation-related pseudoallergy: a new class of drug-induced immune toxicity. *Toxicology* 2005;216:106–21.
- Szebeni J, Baranyi L, Sávy S, Bodó 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* 2006;290:H1050–8.
- Moghimi SM, Hamad I, Andresen TL, Jørgensen K, Szebeni J. Methylation of the phosphate oxygen moiety of phospholipid-methoxy(polyethylene glycol) conjugate prevents PEGylated liposome-mediated complement activation and anaphylatoxin production. *FASEB J* 2006;20:2591–3.
- Szebeni J, Bunger R, Baranyi L, Bedocs P, Toth M, Rosivall L, et al. Animal models of complement-mediated hypersensitivity reactions to liposomes and other lipid-based nanoparticles. *J Liposome Res* 2007;17:107–17.
- Szebeni J, Moghimi SM. Liposome triggering of innate immune responses: a perspective on benefits and adverse reactions. *J Liposome Res* 2009;19:85–90.
- Johnson RA, Simmons KT, Fast JP, Schroeder CA, Pearce RA, Albrecht RM, et al. Histamine release associated with intravenous delivery of a fluorocarbon-based sevoflurane emulsion in canines. *J Pharm Sci* 2011;100:2685–92.
- Cavadas M, González-Fernández, Franco R. Pathogen-mimetic stealth nanocarriers for drug delivery: a future possibility. *Nanomedicine: Nanotechnology, Biology and Medicine* 2011;7:730–43.
- Szebeni J. The interaction of liposomes with the complement system. *Crit Rev Ther Drug Carrier Syst* 1998;15:57–88.
- Szebeni J, Baranyi B, Savay S, Lutz LU, Jelezarova E, Bunger R, et al. The role of complement activation in hypersensitivity to pegylated liposomal doxorubicin (Doxil®). *J Liposome Res* 2000;10:347–61.
- Szebeni J, Bedocs P, Rozsnyay Z, Weiszhar Z, Urbanics R, Rosivall L, et al. Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting

- reactogenicity of Doxil and AmBisome. *Nanomedicine NBM* 2012;8:176–84.
35. Chanan-Khan A, Szebeni J, Savay S, Liebes L, Rafique NM, Alving CR, et al. Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions. *Ann Oncol* 2003;14:1430–7.
  36. Szebeni J, Muggia F, Gabizon G, Barenholz Y. Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention. *Adv Drug Deliver Rev* 2011;63:1020–30.
  37. Hastings KL. Implications of the new FDA/CDER immunotoxicology guidance for drugs. *Int Immunopharmacol* 2002;11:1613–8.
  38. Data Requirements for Intravenous Liposomal Products Developed with Reference to an Innovator Liposomal Product, 2011.
  39. 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.
  40. Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, et al. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 2002;100:1869–77.
  41. Szebeni J, Muggia FM, Alving CR. Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J Natl Cancer Inst* 1998;90:300–6.
  42. Szebeni J, Alving CR, Savay S, Barenholz Y, Prieu A, Danino D, et al. Formation of complement-activating particles in aqueous solutions of Taxol: possible role in hypersensitivity reactions. *Intern Immunopharm* 2001;1:721–35.
  43. Weiszhar Z, Czucz J, Révész C, Rosivall L, Szebeni J, Rozsnyay Z. Complement activation by polyethoxylated pharmaceutical surfactants: Cremophor-EL, Tween-80 and Tween-20. *Eur J Pharm Sci* 2012;45:492–8.
  44. Szebeni J, Baranyi L, Götze O, Alving CR, Bünger R, Mongan PD. Complement activation during hemorrhagic shock and resuscitation in swine. *Shock* 2003;20:347–55.
  45. Szebeni J, Baranyi B, Savay S, Bodo M, Morse DS, Basta M, et al. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. *Am J Physiol* 2000;279:H1319–28.
  46. Boumiza R, Monneret G, Forissier MF, Savoye J, Gutowski MC, Powell WS, et al. Marked improvement of the basophil activation test by detecting CD203c instead of CD63. *Clin Exp Allergy* 2003;33:259–65.
  47. Boumiza R, Debard AL, Monneret G. The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives. *Clin Mol Allergy* 2005;3:9.
  48. Monneret G, Boumiza R, Gendt L, Debard AL. The basophil activation test by flow cytometry: recent improvements and emerging perspectives. *Toxicology* 2005.
  49. Winkler GC. Pulmonary intravascular macrophages in domestic animal species: review of structural and functional properties. *Am J Anat* 1988;181:217–34.
  50. Merkel O, Urbanics R, Bedöcs P, Rozsnyay Z, Rosivall L, Toth M, et al. In vitro and in vivo complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly(ethylene glycol) block copolymers. *Biomaterials* 2011;32:4936–42.
  51. Szebeni J, Bedöcs P, Urbanics R, Bünger R, Rosivall L, Tóth M, et al. Prevention of infusion reactions to PEGylated liposomal doxorubicin via tachyphylaxis induction by placebo vesicles: a porcine model. *J Contr Rel* 2012, doi:10.1016/j.jconrel.2012.02.029.
  52. Szebeni J, Barenholz Y. Complement activation, immunogenicity, and immune suppression as potential side effects of liposomes. In: Peer Pan D, editor. *Handbook of Harnessing Biomaterials in Nanomedicine: Preparation, Toxicity, and Applications*. Stanford Publishing Pte. Ltd., Singapore. 2012:311–35.
  53. Hugli TE, Morgan EL. Mechanisms of leukocyte regulation by complement-derived factors. *Contemp Top Immunobiol* 1984;14:109–53.
  54. Hugli TE. Structure and function of anaphylatoxins. *Spring Semin Immunopathol* 1984;7:193–219.
  55. Marceau F, Lundberg C, Hugli TE. Effects of anaphylatoxins on circulation. *Immunopharmacol* 1987;14:67–84.