

Review

Complement activation-related pseudoallergy: A new class of drug-induced acute immune toxicity

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Abstract

A major goal in modern pharmacotechnology is to increase the therapeutic index of drugs by using nanoparticulate vehicle systems in order to ensure slow release or targeted delivery of drugs. With all great benefits, however, these innovative therapies can carry a risk for acute immune toxicity manifested in hypersensitivity reactions (HSRs) that do not involve IgE but arises as a consequence of activation of the complement (C) system. These anaphylactoid reactions can be distinguished within the Type I category of HSRs as “C activation-related pseudoallergy” (CARPA). Drugs and agents causing CARPA include radiocontrast media (RCM), liposomal drugs (Doxil, Ambisome and DaunoXome) and micellar solvents containing amphiphilic lipids (e.g., Cremophor EL, the vehicle of Taxol). These agents activate C through both the classical and the alternative pathways, giving rise to C3a and C5a anaphylatoxins that trigger mast cells and basophils for secretory response that underlies HSRs. Pigs provide a useful model for liposome-induced CARPA as minute amounts of reactogenic lipomes cause C activation with consequent dramatic cardiovascular and laboratory abnormalities that mimic some of the human symptoms. Consistent with the causal role of C activation in liposome-induced HSRs, a recent clinical study demonstrated correlation between the formation of C terminal complex (SC5b-9) in blood and the presence of HSRs in patients treated with liposomal doxorubicin (Doxil). Overall, the CARPA concept may help in the prediction, prevention and treatment of the acute immune toxicity of numerous state-of-the-art drugs.

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Abbreviations: C, complement; CARPA, complement activation-related pseudoallergy; C1-INH, C1-esterase inhibitor; CrEL, Cremophor EL; HSR, hypersensitivity reaction; MLV, large multilamellar vesicles; PEG, polyethylene glycol; RCM, radiocontrast media; SC5b-9, S protein-bound C terminal complex

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1. Introduction

Hypersensitivity reactions (HSRs) have been traditionally categorized in four groups from I to IV. Coombs and Gell, authors of this concept defined Type I reactions as IgE-mediated acute reactions, while the rest of categories included subacute or chronic immune changes triggered or mediated by IgG, immune complexes or lymphocytes (Coombs and Gell, 1968). 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. Recent estimates suggest that these non-IgE-mediated "anaphylactoid, pseudoallergic or idiosyncratic" reactions may represent as high as 77% of all immune-mediated immediate HSRs (Demoly et al., 1999), implying hundreds of

thousands of reactions and numerous fatalities every year (Szebeni, 2001).

Known examples of pseudoallergy include the reactions caused by radiocontrast media (RCM), nonsteroidal anti-inflammatory drugs, analgetics, morphine and insect venoms, liposomes and micellar solvents, such as Cremophor EL (CrEL) in Taxol. While there is no known common underlying cause for most of these reactions, there is substantial evidence suggesting that the reactions caused by RCM, liposomes and CrEL have a common trigger mechanism: complement (C) activation. Thus, HSRs where the allergen can activate C have been tentatively named C activation-related pseudoallergy (CARPA) (Szebeni et al., 1999, 2000a,b). The phenomenon is increasingly recognized as an immune toxicity issue that has particular significance in the modern field of pharmaceutical nanotechnology; R&D of

Table 1
Symptoms of IgE-mediated Type I allergy and complement activation-related pseudoallergy

Ig-E-mediated Type I	CARPA
Common symptoms	
Angioedema, asthma attack, bronchospasm, chest pain, chill, choking, confusion, conjunctivitis, coughing, cyanosis, death, dermatitis, diaphoresis, dispnoea, edema, erythema, feeling of imminent death, fever, flush, headache, hypertension, hypotension, hypoxemia, low back pain, lumbar pain, metabolic acidosis, nausea, pruritus, rash, rhinitis, shock, skin eruptions, sneezing, tachypnea, tingling sensations, urticaria, wheezing	
Unique symptoms	
Reaction arises after repeated exposure to the allergen	Reaction arises at first treatment (no prior exposure to allergen)
Reaction is stronger upon repeated exposures	Reaction is milder or absent upon repeated exposures
Reaction does not cease without treatment	Spontaneous resolution
Reaction rate is low (<2%)	High reaction rate (up to 45%), average 7%, severe 2%

particulate drug carriers, synthetic nano and microcapsules, liposomes and lipid complexes, micellar carriers and emulsifiers, new formulations of radiopharmaceuticals and contrast agents, etc. (Hunter and Moghimi, 2003; Ten Tije et al., 2003; Storm and Woodle, 2003; Barratt, 2003). This increased awareness of CARPA is also reflected by the fact that testing for C activation in vitro and/or in vivo has become one of the immunotoxicology tests recommended by the US Food and Drug Administration (FDA) that may be useful to identify the pseudoallergy potential of drugs, when needed (Hastings, 2002).

2. Symptoms of CARPA

As listed in Table 1, many symptoms of CARPA are the same as seen in common allergy or classical type I reactions, while others are unique to C activation. Perhaps the most important distinguishing feature of CARPA is the lack of presensitization and reinforcement, i.e., the reaction arises at the first exposure to the drug and then it decreases, rather than increases upon repeated exposure.

3. Complement activation-related pseudoallergy caused by radiocontrast media

3.1. Prevalence and critical factors

Hypersensitivity reactions to RCM, also referred to as “RCM reactions”, have been a concern ever since the first organic, iodinated compound was used for i.v. pyelography in 1928 (Grainger, 2001). Although today, with the use of new-generation RCM, the frequency of severe reactions fell to very low values (see below), the wide use of RCM (in the USA more than 10 million tests are performed yearly (Kumar and Mahalingam, 2001; Hong et al., 2002) still implies a significant number of reactions and occasional fatalities. According to a recent estimate applied for all kinds of symptoms with all procedures and all types of RCM, the overall incidence rate of RCM reactions is 2.1–12.7% (Hong et al., 2002). The frequency of relatively mild cutaneous, vasomotor, pulmonary, cardiovascular or gastrointestinal symptoms is in the 5–8% range (Kumar and Mahalingam, 2001), while life-threatening reactions has been estimated to occur in 0.0004–0.002% of patients, at least in the case of coronary angiography (Kumar and Mahalingam, 2001).

Several factors have been identified to contribute to or influence these reactions, including the osmolality, charge and association of the molecules in RCM, the speed of its i.v. administration and the health, recent

medication and constitutional features of patients. In general, low-osmolality, nonionic, dimeric or trimeric RCM slowly administered to healthy, nonallergic people carries no, or much less risk for HSR than ionic, high-osmolality, monomeric RCM administered as a bolus to people who are recovering from an infection and/or prone for allergy (Hong et al., 2002; Westhoff-Bleck et al., 1990; Barrett et al., 1991; Katayama et al., 2001; Henry et al., 1991).

3.2. Pathomechanism of RCM reactions

The pathogenesis of RCM reactions is considered multifaceted, as beside C activation, several other mechanisms and controlling factors were shown to play more or less roles. As illustrated in Fig. 1, mast cells and basophils are in the centre of RCM reactions. They can be triggered by RCM molecules directly, through poorly understood intracellular interactions and/or extracellular physical effects (for example, osmotic stress), or indirectly, via cell membrane receptors. The latter include the FcεR and the anaphylatoxin receptors (C5aR and C3aR), binding IgE, C5a and C3a, respectively. Positive feedback can be provided by co-activation of the coagulation and kinin-kallikrein systems, leading to crossover activation of the C cascade with depletion of C1INH

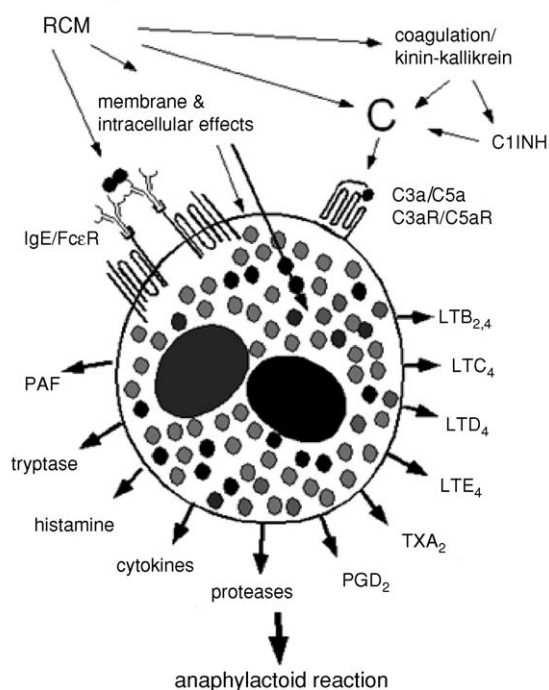


Fig. 1. Schematic representation of the multiple factors and effects involved in RCM reactions. Taken from (Szebeni, 2004a,b) with permission of the publishers.

(Szebeni, 2001, 2004a,b; Fattori et al., 1992). The negative control mechanisms include all those factors and effects that suppress the activation of the C and other plasma proteolytic systems (factors I, H, C1Inh, C4bp, etc.) (Mollnes, 2004).

As for the effector arm of RCM reactions, secondary mediators include histamine, tryptase, PAF, LTB₂, LTB₄, LTC₄, LTD₄, LTE₄, TXA₂, PGD₂ and TXD₄ (Westhoff-Bleck et al., 1990; Greenberger, 1984; Lieberman, 1991) (Fig. 1). Some of these mediators (e.g., PAF, histamine, tryptase and TXA₂) are preformed and liberate from the cells immediately upon activation, while others are de novo synthesized and, hence, liberate slower. Further variations of RCM reactions are due to individual differences in the expression of cellular receptor subtypes on which mast cell secretion products act. For example, activation of H1 receptors leads to vasoconstriction and vascular leakage, and is responsible for the cardiovascular and cutaneous symptoms of anaphylaxis. H2 receptors, on the other hand, increase cellular cAMP levels and cause vasodilation, increased heart rate and pulse pressure (Lieberman, 1989).

Another important factor in the secretory response of mast cells and basophils to RCM is the location of these cells. For example, mast cells from the skin may not respond to certain RCM, while pulmonary and cardiac mast cells are triggered for strong release reaction (Genovese et al., 1994). Likewise, mannitol, via osmotic stimulus, may induce the release of histamine from human basophils, but to a lesser extent from mast cells (Genovese et al., 1994).

3.3. Complement activation as underlying cause of RCM reactions

RCMs have a complex impact on the C system mainly via physical effects (charge, viscosity, iodine number, hydrophilicity and osmotic pressure) (Lieberman, 1991; Napolovlu et al., 1998; Vik et al., 1995). Complement activation by RCM was demonstrated to proceed both through the classical and the alternative pathways (Lieberman, 1991; Napolovlu et al., 1998), as well as via uncommon mechanisms, such as non-localized, non-sequential cleavage of C proteins (Kolb et al., 1978), suppression of Factors H and I (Lieberman, 1991) and direct action on the thioester bonds of C4 and C3 (Vik et al., 1995).

Among the animal studies attesting to a causal role of C activation in RCM reactions Lasser et al. reported severe “idiosyncratic” response of a dog to the injection of sodium iohalamate, manifested in vomiting, hypotension and hyperreflexia. The authors

found significant depletion of C during the symptoms, suggesting that C activations was causally involved in the reaction (Lasser et al., 1976). In further dog studies by Lang et al. serial daily injections of RCM (metrizamide, iohalamate, diatrizoate, acetrizoate, iodipamide and iopanoate) caused substantial declines of serum C over several days (Lang et al., 1976). In rats, Napolovlu et al. proved that various RCM in the 0.5–2.0 g iodine/kg range activated the C system via the alternative pathway, with efficacy in the following order: triombrast > hexabrics > ultravist ≥ melitracast = omnipac (Napolovlu, 1997; Napolovlu et al., 1998).

In humans, case reports implicating C activation in more or less severe RCM reactions gave account of decreased plasma hemolytic C, C3, C4 (Lasser et al., 1980), decreased factor B and C1 esterase inhibitor (C1INH) levels (Lasser et al., 1980; Vandenplas et al., 1990), rises of C3 conversion products (Lasser et al., 1980; Vandenplas et al., 1990) and the presence of consumption coagulopathy (Lasser et al., 1980; Vandenplas et al., 1990), pulmonary capillary leakage (Vandenplas et al., 1990) and acute respiratory distress syndrome with granulocytic aggregates in the pulmonary microcirculation (Lieberman, 1991).

Among the more extensive clinical studies looking at the role of C activation in RCM reactions, Small et al. (1982) analyzed HSRs and C activation in 220 patients undergoing i.v. pyelography. Nineteen percent of patients displayed HSRs, while depressed serum CH₅₀ levels, indicating C activation, occurred in 49%. The RCM-induced decline of CH₅₀/mL was apparent within 90 s after starting the infusion and returned to normal after about 30 min. This study highlighted an important fact regarding the relationship between C activation and HSRs, namely, that more people display signs of C activation than HSRs. Hence, C activation may be present in patients without clinically manifest reaction, suggesting that anaphylatoxin liberation does not necessarily cause HSRs. C activation may therefore be a precondition, or contributing factor to HSRs, but it does not solely explain the phenomenon. Other factors or preconditions may also need to be present in people who develop HSR. This point was reinforced in the studies by Westaby et al. (1985), who demonstrated significant elevation of the anaphylatoxin C3a in the peripheral blood of 7/11 patients receiving RCM for coronary angiography. In 3/7 patients, C3a was increased between 4- and 10-fold, yet only one of these patients developed symptoms, which were mild.

It should be noted that C activation has not been a consistent finding in all clinical studies reporting C measurements in patients injected RCM. Kolb et

al. (1978), for example, found no significant changes in CH_{50} and hemolytic C3 activity in serum samples obtained from 40 patients before and 30 min after undergoing i.v. pyelography with methylglucamine diatrizoate or iothalamate.

4. Complement activation-related pseudoallergy caused by drug carrier liposomes and lipid complexes

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 advanced clinical trials, or already used in patients mainly for anticancer and antifungal applications (Szebeni, 2004a,b). Out of the marketed liposomal drugs Doxil (Caelyx) (Uziely et al., 1995; Alberts and Garcia, 1997; Dezube, 1996; Gabizon and Martin, 1997; Gabizon and Muggia, 1998; Chanan-Khan et al., 2003), AmBisome (Levine et al., 1991; Laing et al., 1994; Ringdén et al., 1994; de Marie, 1996; Schneider et al., 1998), Abelcet (de Marie, 1996), Amphocil (de Marie, 1996) and DaunoXome (Cabriaes et al., 1998; Eckardt et al., 1994; Fossa et al., 1998; Gill et al., 1995, 1996; Girard et al., 1996; Guaglianone et al., 1994; Money-Kyrle et al., 1993; Richardson et al., 1997) have been reported to cause HSRs with symptoms corresponding to CARPA (Table 1). The frequency of HSRs to liposomal drugs shows large variation between 3 and 45% (Szebeni, 1998, 2001).

4.1. Evidence for a role of C activation in liposome reactions

4.1.1. *In vitro* studies

Since its discovery in the late sixties (Haxby et al., 1968; Alving et al., 1969), C activation by liposomes has been analysed in a great number of studies. The emerging picture is very complex, as variations in liposome structure and other experimental conditions can result in fundamental differences in the extent, pathway and kinetics of activation (Szebeni, 2001, 1998).

Focusing on C activation by Doxil, as an example, its incubation with 10 different normal human sera led to significant rises in C terminal complex (SC5b-9) levels over PBS control in seven sera, with rises exceeding 100–200% (relative to PBS control) in four subjects (Szebeni et al., 2000b). Further experiments showed that in addition to the quantitative variation in SC5b-9 response, Doxil-induced C activation also varied in different individuals in terms of sensitivity to inhibition by

10 mM EGTA/2.5 mM Mg^{2+} , which distinguishes classical from alternative pathway activation (Szebeni et al., 2000b). The minimum effective C-activating concentration of Doxil was 0.05–0.10 mg/mL and there was near linear dose–response relationship up to about 0.5 mg/mL. The activation curve reached plateau at doses ≥ 0.6 mg/mL, suggesting saturation of response (Szebeni et al., 2000b). Doxil also caused variable liberation of Bb, a specific marker of alternative pathway activation, providing further evidence for a role of alternative pathway activation and/or amplification (Szebeni et al., 2000a,b).

These and other studies from our laboratories (Szebeni et al., 1994, 1996, 1997a,b, 1999, 2000a,b, 2002; Szebeni and Alving, 1999) highlighted some basic conditions and mechanism of liposomal C activation. Thus, large size, polydispersity, positive or negative surface charge and high (>45%) cholesterol content were all shown to promote, whereas small uniform size and neutrality reduced the proneness of liposomes for C activation. The process may involve both the classical and alternative pathways, with the latter acting either as the only activation mechanism, or as a positive feedback mechanism amplifying C activation via the classical pathway. As for the classical pathway, the presence of liposome-reactive immunoglobulins represents a powerful trigger or enhancer, but their presence is not a precondition for C activation via this pathway. Direct binding of C1q to the phospholipid bilayer, or to C reactive protein-tagged liposomes, can also activate C via the classical pathway. Thus, C activation by liposomes can involve numerous redundant triggering and controlling processes whose differential manifestations in individuals may explain, at least in part, the substantial variation of *in vivo* responses to liposomes, as discussed below.

4.1.2. Clinical evidence for C activation as underlying mechanism of liposome-induced HSRs

The likely clinical relevance of C activation by liposomes can be deduced from clinical studies in the past, taken together with a recent study dedicated to address the cause–effect relationship between *in vivo* C activation and clinical reactions to Doxil.

In reviewing the historic evidence, one of the earliest clinical studies with liposomal drugs reported that intravenous infusion of vesicles containing NSC 251635, a water-insoluble cytostatic agent, led to increased C3d/C3 ratios in the plasma of cancer patients (Coune et al., 1983). Another, also still indirect proof, is the finding of Skubitz et al. (Skubitz and Skubitz, 1998) on transient neutropenia with signs of leukocyte activation in patients who displayed HSRs to Doxil. As is known, neutropenia with leukocyte activation are classical hallmarks of ana-

phylatoxin action (Cheung et al., 1994; Skroeder et al., 1994a,b).

To the authors' knowledge the first direct evidence for the causal relationship between C activation and HSRs to liposomes was provided by Brouwers et al. (2000), who reported 3 severe HSRs out of 9 patients obtaining ^{99m}Tc -labeled pegylated liposomes for scintigraphic detection of bowel inflammation (Dams et al., 2000). In one reactor patient plasma C3, C4 and factor B decreased by 16–19%, implying major C consumption. The fact that both C4 and factor B were depleted suggests that C activation involved both the classical and the alternative pathways.

The study dedicated to correlate C activation with HSRs gave account of 45% reaction rate in cancer patients infused with Doxil for the first time (Chanan-Khan et al., 2003). The grade 2 or 3 HRSs in 13 of 29 patients occurred in men and women in approximately equal proportions and were not related to the age of patients. Importantly, Doxil caused C activation in 21 out of 29 patients (72%) as reflected by significant elevations of plasma SC5b-9 levels following infusion of the drug. The time course of SC5b-9 increase in blood showed substantial individual variation (Fig. 2), includ-

ing rapid elevations within 10 min with gradual return to near baseline within 2 h (A), rapid elevation without return within 2 h (B) and moderately rapid elevation of SC5b-9 until about 30 min, followed by partial return to baseline during 2 h (C). The lack of SC5b-9 response is demonstrated in Fig. 2D.

Taking the baseline and 10 min post-infusion SC5b-9 values in clinical reactors and non-reactors, the study reported significant increase of SC5b-9 in 12/13 reactor patients in contrast to 9/16 in the non-reactor group (Fig. 3). Thus, 92% of clinical reactors were also laboratory reactors, while only 56% of clinical non-reactors were laboratory reactors. These data led to the conclusion that C activation and HSR show significant ($P < 0.05$) correlation.

The quantitative relationship between SC5b-9 values at 10 min and severity of HSR revealed that the SC5b-9 assay is highly sensitive in predicting HSRs (Table 2), although the specificity and positive predictive value of the test was relatively low, particularly in patients in whom the rise of SC5b-9 at 10 min remained below two-times the upper limit of normal SC5b-9 (Table 2, row 2). However, restricting the criteria for laboratory reactivity to 10 min SC5b-9 values exceed-

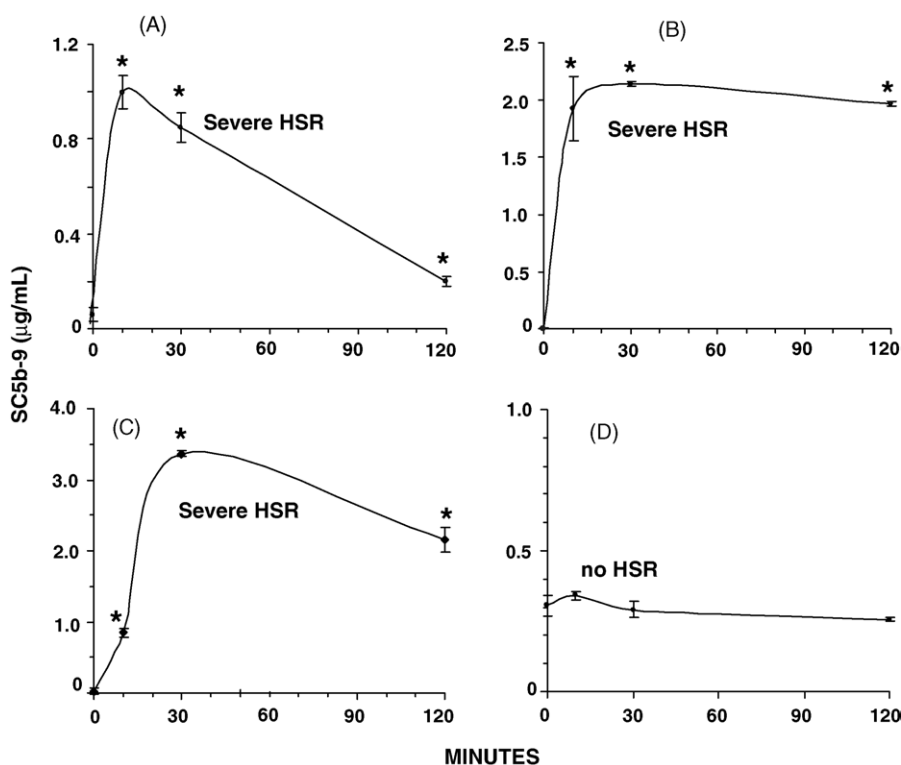


Fig. 2. Time course of Doxil induced changes in plasma SC5b-9 in cancer patients and its individual variation. Panels A–D demonstrate data from four subjects displaying different patterns of response. Data are mean \pm S.D. for triplicate determinations. (*) Significantly different from baseline, $P < 0.05$. Reproduced from (Chanan-Khan et al., 2003) with permission.

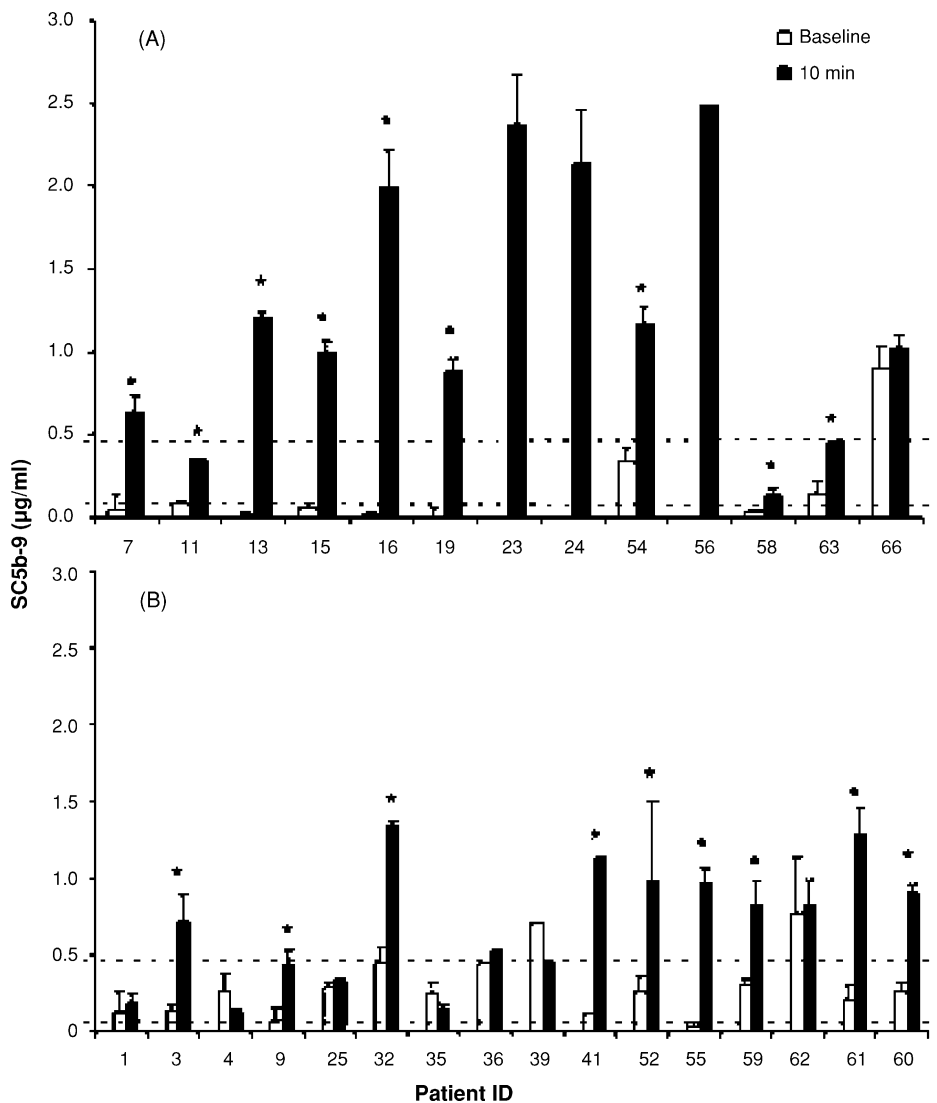


Fig. 3. Plasma SC5b-9 levels at baseline and at 10 min post-infusion of Doxil in cancer patients displaying (A) or not displaying (B) HSRs to Doxil. Data are mean \pm S.D. for triplicate or duplicate determinations. The dashed lines indicate the normal range of SC5b-9, i.e., the normal mean \pm 2 S.D. (*) Significantly different from baseline ($P < 0.05$). The numbers under the bars are the patient ID. Reproduced from (Chanan-Khan et al., 2003) with permission.

ing two- or four-fold the upper threshold of normal (Table 2, row 3), the specificity and positive predictive value of the C assay remarkably increased with relatively less decrease in sensitivity. Thus, the extent of SC5b-9 elevation was proportional with the specificity and positive predictive value of the C assay with regards to HSRs.

Finally, the study revealed significant correlation between dose rate and SC5b-9 ($P < 0.01$), indicating that C activation at 10 min was Doxil dose dependent.

In summary, these data strongly suggested that C activation might be causal or a key contributing, but

not rate-limiting factor in liposome-induced CARPA. This proposal is keeping with the mentioned report by Small et al. (1982) wherein 19 % of patients infused with a RCM displayed HSR, although C activation was detectable in 49%. A plausible hypothesis explaining the phenomenon is that reactors differ from nonreactors in at least two criteria: (1) they are susceptible for C activation by the drug and (2) their mast cells and basophils have a lower than normal threshold for secretory response to anaphylatoxins. Consistent with this idea, proneness for HSRs is known to correlate with the presence of other allergies, i.e., with atopic constitution. Hence, the risk of

Table 2
The SC5b-9 assay as predictor of HSRs to Doxil

10 min SC5b-9 ($\mu\text{g/mL}$)	Sensitivity tp/(tp + fn)	Specificity tn/(fp + tn)	Positive predictive value tp/(tp + fp)	Negative predictive value tn/(fn + tn)
Significant increase* (SC5b-9, no limit)	0.92	0.44	0.57	0.88
Significant increase* SC5b-9 ≤ 0.98	0.83	0.54	0.45	0.88
$0.98 \leq \text{SC5b-9} \leq 1.96$ ($\geq 2\times$, $\leq 4\times$ normal)	0.80	0.70	0.57	0.88
SC5b-9 ≥ 1.96 ($\geq 4\times$ normal)	0.75	1.00	1.00	0.88

Patients were classified into four groups according to the concurrent presence (+) or absence (–) of HSR and C reactivity, as follows: true positive (tp: HSR+, C+), false positive (fp: HSR–, C+), true negative (tn: HSR–, C–) and false negative (fn: HSR+, C–). In addition, laboratory reactors were stratified to three categories on the basis of 10 min SC5b-9 values, as specified in column 1. The 0.98 and 1.96 $\mu\text{g/mL}$ cut-off values represent two- and four-times the upper limit of normal SC5b-9 levels (0.49 $\mu\text{g/mL}$), respectively, and were chosen arbitrarily. The sensitivity, specificity and positive and predictive values of the SC5b-9 assay with regard to HSRs were computed as described (Chanan-Khan et al., 2003).

* Significant increase refers to significant ($P < 0.05$) increase of 10 min SC5b-9 relative to baseline. Reproduced from (Chanan-Khan et al., 2003) with permission.

CARPA may be highest in those atopic subjects who are also sensitive to C activation.

5. Role of C activation in HSRs to Cremophor EL and other solvent systems containing amphiphilic emulsifiers

The third group of intravenous agents causing CARPA is characterized by the presence of an amphiphilic emulsifier in the infusion liquid, such as the semisynthetic Cremophor EL (CrEL), emulphor (Blum et al., 1979; O'Dwyer and Weiss, 1984; Athanassiou et al., 1988) or synthetic block copolymers, such as poloxamer 188.

5.1. Cremophor EL

Cremophor EL has been used to solubilize many water insoluble drugs, including paclitaxel (Taxol), cyclosporine, the antineoplastic agents Teniposide, Echinomycin and Didemnin E, the anaesthetic agents propanidid and althesin, steroids and vitamins (A, D, E, K) (Lassus et al., 1985). It is a non-ionic detergent, a complex mixture of unmodified castor (ricinus) oil and a large variety of polyethylene glycols and amphiphilic polyethoxylated glycerols, polyethoxylated fatty acids (mostly ricinoleic acid) and polyethoxylated glycerol esters differing in acyl chain and/or polyethyleneoxide length (Szebeni et al., 1998, 2001). While all the above-mentioned drugs dissolved in CrEL have been reported to cause HSRs (Lassus et al., 1985; Nolte et al., 1988) the best-known manifestation of hypersensitivity to CrEL is the reactions caused by Taxol, one of the best-known anticancer drugs today.

Reactions to Taxol were reported soon after the beginning of Taxol trials in the early eighties (Kris et al., 1986) and kept attracting attention ever since, as 2–7%

of patients still develop HSRs despite their intense pre-medication with corticosteroids (mostly dexamethason) and antihistamines (diphenhydramine, cimetidine, ranitidine) (Rowinsky et al., 1991, 1992, 1994; Rowinsky, 1993, 1997; Rowinsky and Donehower, 1995; Guchelaar et al., 1994; Bookman et al., 1997a,b; Ramanathan et al., 1996; Essayan et al., 1996; Nannan Panday et al., 1997; Grosen et al., 2000). These reactions are severe, life-threatening in up to 1–3% of patients with occasional deaths mostly due to cardiac arrest. Fitting in the CARPA category (Table 1), HSRs to Taxol usually arise within minutes after starting the infusion and include common, as well as unusual allergic symptoms that are explainable with C activation. In addition to the patients' difficult time, the unpredictability and often dramatic picture of Taxol reactions cause substantial anxiety for doctors and caretakers, and they also represent a significant logistic and financial burden on hospitals, the latter estimated to be US\$ 2900–4900/cycle (Grosen et al., 2000). Most importantly, these reactions exclude some 2% of patients from obtaining a therapy that is considered as state-of-art in extending life in certain cancers.

Although it has been early recognized that the above HSR to Taxol is due to CrEL, this toxic vehicle was not replaced by the manufacturer (Bristol-Myers) for probably numerous reasons, including logistic, economic and scientific. The scientific rationale might have been based on the concept that CrEL might contribute to the antitumor efficacy of Taxol by a suppressive influence on P-glycoprotein-mediated multidrug resistance (Webster et al., 1993; Kessel et al., 1995). Nevertheless, other groups and drug companies have embarked on intense research and development of non-toxic vehicles for paclitaxel (Terwogt et al., 1997; Lundberg, 1997; Paradis and Page, 1998; Scripture et al., 2005), with AbraxaneTM (American Pharmaceutical Partners Inc. and American Bioscience Inc.) becoming the first

CrEL-free formulation approved by the FDA for the treatment of metastatic breast cancer. AbraxaneTM, an albumin nanoparticle-based paclitaxel formulation, was reported to cause less HSRs compared to traditional Taxol, despite the fact that it was given to patients without steroid and antihistamine premedication, at 50% higher dose and shorter infusion time (Garber, 2004; Sparreboom et al., 2005).

5.1.1. Complement activation as underlying cause of CrEL toxicity

The concept that C activation by CrEL would underlie HSRs to Taxol was based on the demonstration that CrEL fully accounted for C activation by Taxol *in vitro* (Szebeni et al., 1998). Both Taxol and an equivalent amount of CrEL caused significant elevation of SC5b-9 and Bb in the sera of normal as well as cancer patients following incubation with therapeutically relevant concentrations. This C activation could be inhibited by soluble C receptor type 1 (Szebeni et al., 1998). As for the mechanism of C activation by CrEL, we considered that CrEL is a non-ionic emulsifier consisting of a mixture of amphiphilic molecules that form micelles in water (Kessel, 1992; Nerurkar et al., 1997; Trissel, 1997). Micelles are multimolecular aggregates in the nanometer size range, and those formed from amphiphilic polymers usually appear as spherical “core-shell” structures with a dense nucleus surrounded by a less electrode dense halo (insert in Fig. 4A) (Kwon, 2003). Thus, micelles represent a particulate substance unprotected by surface-bound C regulatory proteins (e.g., CR1, DAF, MCP), therefore satisfying two basic conditions for becoming a C activator (Liszewski and Atkinson, 1993). It was, however, unclear whether micelles are present in Taxol solutions under the conditions of infusion therapy, and even if the answer is yes, whether they could cause C activation *in vivo*?

To address the former question, we utilized various physicochemical and imaging techniques to explore and characterize particles in clinically relevant aqueous solutions of Taxol (Szebeni et al., 2001). As shown in Fig. 4A and B, cryo-transmission electron microscopy (Cryo-TEM) showed 8–20 nm spherical structures in aqueous solutions of Taxol mimicking the drug infused in patients, typical “core-shell”, also called “star” micelles that are formed from amphiphilic polymers (Kwon, 2003). As for the question, whether these micelles can cause C activation, we demonstrated that filtration of aqueous solutions of Taxol or pure CrEL via 30 kDa cut-off filters eliminated, while the filter retentate restored the potentiating effect of these agents on SC5b-9 formation in human serum (Szebeni et al., 2001). Thus, the

effect was due to particles with MW > 30 kDa, which is consistent with a causal role of micelles.

However, there was still a problem with the implication of micelles in C activation, considering that their dimension is comparable to the classical and/or alternative pathway C3 convertases (for example, C3bBb is about 14 nm × 8 nm (Smith et al., 1982)). Specifically, the surface of even the largest, ~20 nm micelle appears to be too small to allow deposition of C3 convertases, at least as it occurs in the case of C activating cell membranes and other surfaces. A possible solution to this puzzle was provided by our observation (Szebeni et al., 2001) that CrEL micelles underwent massive structural transformation in human plasma, forming microdroplets of varying size up to about 300 nm (Fig. 4C and D). Although we had no experimental data supporting the claim that these microdroplets were in fact formed from CrEL micelles, a previous study by Kessel et al. (1995) provided strong indirect support for this proposition. Namely, in studying the effect of Taxol on plasma lipoproteins in cancer patients, these authors noted an increase in size, as well as a decrease in the electrophoretic mobility of HDL and/or LDL relative to pre-treatment values. They also noted that the originally sharp HDL and LDL bands became smeared, and that a new, highly sudanophilic band was formed which slightly migrated towards the cathode (Kessel et al., 1995). Clearly, CrEL and plasma lipoproteins underwent substantial interaction, which is not surprising in light of the fact that CrEL is a mixture of lipids. In particular, the above data suggest incorporation of CrEL lipids into HDL and LDL, as well as the formation of positively charged particles from apolar molecules in CrEL that did not associate with lipoproteins. Based on these data we suggested that some of the dense, small structures in our cryo-TEM image of human plasma incubated with Taxol (Fig. 4C and D) are CrEL enriched, enlarged lipoproteins, whereas the large lipid droplets may correspond to the newly formed structures composed of hydrophobic CrEL molecules with some charged amphiphilic components at the water–oil interface.

As for the question, how these newly formed CrEL particles in blood activate C, one possibility is that they bind C3 in a fashion similar to that described for the nonionic block copolymer surfactants, L101 and L121 (Szebeni et al., 1998). The latter particles were shown to activate C via the alternative pathway, due to the binding of C3 to their hydrophilic adhesive surface (Hunter and Bennett, 1984, 1987; Hunter et al., 1994). In strong support of this proposal, the length of the hydrophilic (polyethoxylated) chains in many amphiphilic molecules in CrEL is remarkably similar to those found in L101

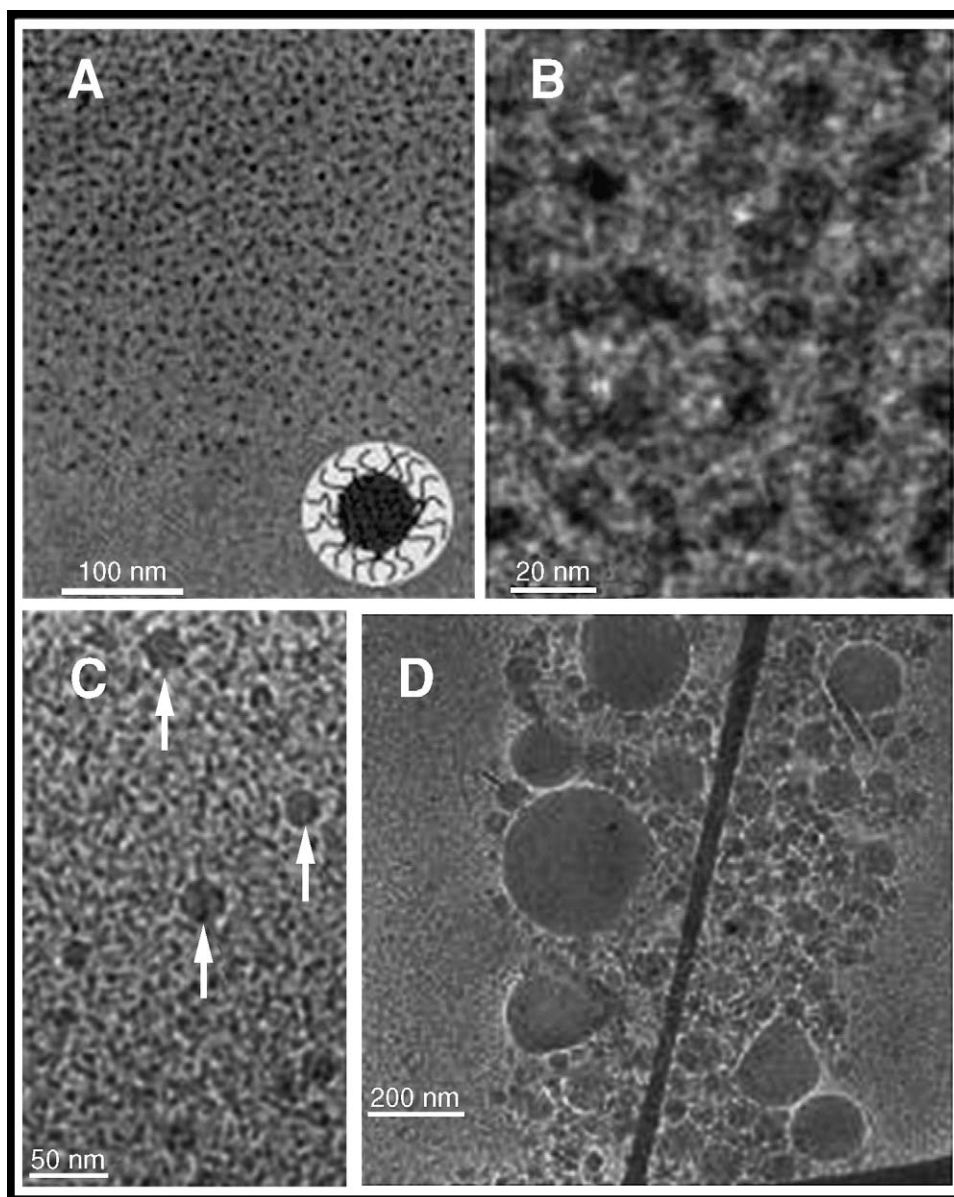


Fig. 4. Cryo-TEM images of vitrified specimens of Cremophor EL in saline (PBS) and in human serum. (A) Taxol vial-equivalent CrEL/ethanol stock solution was diluted 10-fold in PBS. The dark spots represent “star” micelles, schematically depicted in the insert. (B) Larger amplification of CrEL micelles. (C) Vitrified specimens of a normal human serum, demonstrating some lipoprotein particles in the chylomicron size range. (D) CrEL was incubated with the same serum for 10 min at 37 °C, leading to the formation of numerous particles of varying size. Reproduced from (Szebeni et al., 2001) with permission.

(Szebeni et al., 1998). Furthermore, positively charged particles (liposomes) were reported to induce C activation via the alternative pathway (Chonn et al., 1991).

5.2. Synthetic amphiphilic polymers

Just as the semisynthetic emulsifier molecules discussed above, synthetic amphiphilic polymers have also

been used as solvent systems for water insoluble drugs. Some of these polymers have also been used as vaccine adjuvants or as pharmacokinetics-modifier drug conjugates. Examples for synthetic amphiphilic polymers include poloxamers, poloxamines, other copolymers of hydrophilic and hydrophobic blocks, such as polyoxyethylene, polyoxypropylene and polyethyleneglycols (PEG) attached to phospholipids or to low molecular

acyl chains (Kwon, 2003). These solvents can also cause HSRs, the best-known example of which is the reaction to poloxamer 188, also known as Pluronic F-68, used as an additive in Fluosol DA, a perfluorocarbon-based blood substitute (Vercellotti et al., 1982).

As for the mechanism of poloxamer-induced C activation, Vercellotti et al. found that the Fluosol DA-induced C3 conversion, C consumption and C5a generation in rabbit plasma was prevented by EDTA, but not by EGTA, suggesting alternative pathway C activation (Vercellotti et al., 1982). The involvement of the alternative pathway in poloxamer 188-induced C activation in human plasma was confirmed in a recent study by Moghimi et al. as well (Moghimi et al., 2004). The latter study also provided evidence that poloxamer 188-mediated C activation is an intrinsic property of the polymer, and that it is independent of the degree of sample polydispersity or trace amounts of nonpolymeric contaminants in the preparation, such as organic volatiles (acetaldehyde and propionaldehyde). C activation was triggered at submicellar concentrations of the polymer and was partially due to the presence of double bonds therein. Consistent with the idea that an interaction with plasma lipoproteins plays a key role in polymer-induced C activation, quasi-elastic light scattering established major changes in lipoprotein size following the addition of poloxamer to plasma (Moghimi et al., 2004). However, poloxamer-induced rise in SC5b-9 was significantly suppressed when serum HDL and LDL cholesterol levels were increased above normal levels, suggesting that lipoprotein binding can impact C activation by poloxamer in a complex, dose-related fashion (Moghimi et al., 2004).

6. Animal models of CARPA

6.1. Porcine model

Swine are particularly sensitive for liposome-induced cardiopulmonary distress, a feature that may be related to the presence of pulmonary intravascular macrophages in this, as well as in other ungulate (Artiodactyla) species (Winkler, 1988). Over the past 6 years or so, we have injected a total of 105 pigs with various liposomes. Without exception, minute amounts (5–10 mg) of multilamellar vesicles (MLV, consisting of dimyristoylphosphatidylcholine, dimyristoylphosphatidylglycerol and cholesterol, 45:5:50 mole ratios) or equal amounts of zymosan caused major hemodynamic changes as detailed below. With liposomes other than MLV the reaction was variably present or absent depending on the phospholipid composition, size, charge and encapsu-

lated material within the vesicles (Szebeni et al., 1999, 2000a,b, 2002). Interestingly, CrEL did not induce cardiopulmonary distress in pigs even in large (hundreds of milliliters) amounts (unpublished observations). We hypothesize that this unresponsiveness could be due to atypical of ineffective alternative pathway activation (by CrEL) in pigs.

The hemodynamic changes caused by liposomes in pigs include massive rises in pulmonary arterial pressure (PAP) with declines of systemic arterial pressure, cardiac output and left ventricular end-diastolic pressure (Szebeni et al., 1999, 2000a,b). The hemodynamic changes were associated with massive, although transient ECG alterations including tachycardia, bradycardia, arrhythmia, ST segment and T wave changes, ventricular fibrillation and cardiac arrest, all attesting to severe myocardial ischemia and consequent functional disturbance (Szebeni et al., 2005).

C activation-related pulmonary hypertension in pigs was highly reproducible, quantitative and specific. The high reproducibility of the reaction is illustrated by the remarkably low variation in the rise of PAP in response to a same dose of liposomes (Szebeni et al., 1999). The quantitative nature of this “large animal bioassay” was shown by the linear relationship between liposome dose and submaximal rises of PAP (Szebeni et al., 1999), whereas its specificity to C activation became evident from the observations that (1) small unilamellar liposomes, which had negligible C activating effect *in vitro*, also failed to cause hemodynamic changes *in vivo* (Szebeni et al., 2000a,b) and (2) non-liposomal C activators (zymosan, xenogeneic immunoglobulins) induced pulmonary pressure changes that were indistinguishable from those caused by MLV (Szebeni et al., 1999).

Considering that (1) hypotension is one of the major symptoms of acute HSRs to liposomes in patients; (2) pulmonary hypertension with consequent preload reduction (decrease of left ventricle filling with coronary hypoperfusion) can explain the dyspnea with chest and back pain in man; (3) the ECG changes observed in the pigs mimic the cardiac electric abnormalities reported in HSRs to liposomes (Ambisome) (Aguado et al., 1993); (4) the vasoactive dose of Doxil in pigs in the 0.02–1 mg/kg range corresponds to the dose that triggers HSR in humans (Gabizon and Muggia, 1998), we proposed that pigs provide a sensitive model of those human subjects who display HSR to liposomes (Szebeni et al., 1999, 2000a,b). As mentioned, the US drug regulatory agency (FDA) recommended consideration of C activation among the immune toxicology tests, when necessary (Hastings, 2002).

6.2. Dog model

While the hemodynamic response of dogs to liposomes is less dramatic than that in pigs, dogs do develop pronounced blood cell alterations in response to liposomes or other C activators (unpublished data). Interestingly, dogs also display considerable vegetative neural dysfunction during HSRs (hyperreflexia, diarrhea, vomiting, extensive salivation), a phenomenon that may represent a unique interaction between the immune and neural systems in this species. As for the hemodynamic changes, it is important to point out that dogs are prone for histamine reactions, and can develop major HSRs without involvement of the C system, as seen with CrEL (Lorenz et al., 1977).

7. Clinical testing of CARPA

The above discussed study of Chanan-Khan et al. (2003), wherein the symptoms of Doxil reactions were quantified and correlated with plasma SC5b-9, may provide guidelines and further ideas for future clinical studies testing the CARPA concept. In particular, the findings highlight the need for measuring other C cleavage products in addition to SC5b-9, as the latter is only an indirect measure of C5a production. It cannot be excluded that some clinical reactors with no elevated SC5b-9, who were considered “false negative”, actually produced increased amounts of C3a (and/or C5a). Possibly useful C activation assays, in addition to SC5b-9, include C3a-desarg, C5a-desarg, Bb, iC3b and C4d ELISAs (Szebeni et al., 2003).

Based on the porcine model (Szebeni et al., 1999, 2000a,b), a further recommended extension of the Chanan-Khan et al. (2003) protocol is continuous heart rate, blood pressure and ECG monitoring during the initial phase of the infusion of pseudo-allergenic drugs. It is possible that subclinical hemodynamic changes will be present in laboratory reactor but clinical non-reactor “false positive” patients, thereby strengthening the relationship between C activation and clinical reaction. Yet another possible monitoring technique is the measurement of (skin) conductance, a principle that was applied in a recent report of rheoencephalographic detection of cerebrovascular changes during CARPA in pigs (Bodo et al., 2005).

An essential conclusion of the Chanan-Khan et al. (2003) study was that C activation could be a precondition, but not rate limiting factor in CARPA, thus additional processes are likely to play causal and/or controlling roles in the clinical response. The patient's proneness for mast cell activation by anaphylatoxins

represents a likely rate limiting step, thus, in principle, the demonstration of increased susceptibility to C activation and/or to C-mediated mast cell (basophil) activation can provide mutually supportive evidence for CARPA. There are many versions of the basophil activation assays detecting surface markers (CD63 or CD203c, Boumiza et al., 2003) and/or secretory products (histamine, tryptase, eicosanoids, cytokines, Szebeni, 2001). When assaying CARPA it is essential to use whole serum or non-EDTA anti-coagulated plasma with minimal dilution, as the interaction of C with the cells needs to mimic the physiological conditions. It is also important to emphasize that the use of fresh serum or plasma is preferential in these assays, as repeated thawing inactivates C (Chanan-Khan et al., 2003). Also, because C activation proceeds on a time scale of minutes, the time window of blood sampling and *in vitro* basophil activation should cover the first few minutes.

These straightforward principles and relatively simple assays for the clinical testing of CARPA will hopefully be considered by experimenters in the cancer and/or radiology field, who wish to explore the molecular basis and novel therapies of pseudoallergic drug reactions.

8. Theoretical implications

Apart from better understanding the pathomechanism of HSRs, the CARPA concept may represent a step forward in solving a major dilemma in theoretical immunology; the classification of HSRs. Gell and Coomb's system of four categories (types I–IV) has serious limitations, including the fact that pseudoallergy cannot be fitted in any of the four types of HSRs. However, no consensus has been reached to date, how to replace this classification. Descotes and Choquet-Kastylevsky (2001) proposed the use of three major types, namely, pseudoallergy, immunoglobulin-mediated and cell-mediated HSRs. Aronson and Ferner (2003) suggested to specify and graphically characterize HSRs according to their time course, susceptibility and dose dependence. The author or this review (Szebeni, 2001) laid out a functional categorization that differentiates acute (Type I) HSRs 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 ϵ receptor mediated, (b) anaphylatoxin-triggered and C3a/C5a receptor-mediated “CARPA” and (c) mixed type reactions, triggered by both IgE and anaphylatoxins (Fig. 5). This classification covers many previously uncategorized, C-mediated reactions that arise upon the

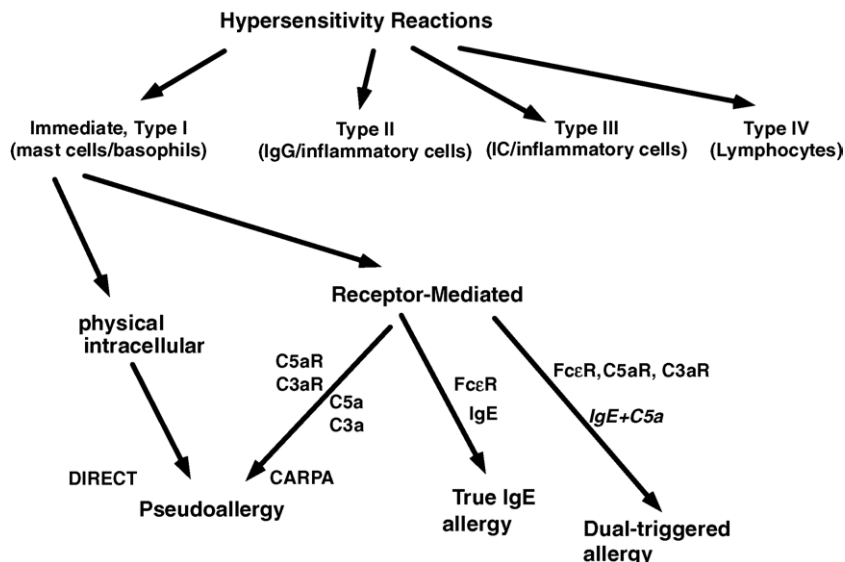


Fig. 5. Proposed new scheme of hypersensitivity reactions with Revision of the Type 1 category. Partially reproduced from (Szebeni et al., 2002).

use of extracorporeal circuits, RCM and various liposomal and micellar carriers of intravenous drugs.

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