Short Communication

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Paradoxical rise of hemolytic complement in the blood of mice during zymosan- and liposome-induced CARPA: a pilot study

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Abstract: The complement (C) activating effect of zymosan and liposomal drugs (AmBisome, Caelyx) leads to significant C consumption in rats, dogs, pigs and other species in vivo, as reflected by a fall in hemolytic complement activity (HCA) of their plasma. However, the acute C activating effect of zymosan and liposomal drugs is unclear in the mouse. Therefore, using sheep red blood cells, we assayed the HCA of plasma obtained from apolipoprotein E-deficient (ApoE) as well as from background C57BL/6 (BL6) mice. Intravenous (i.v.) administration of C activators led to a significant rise (up to 40%) in HCA of the plasma. The HCA steadily rose up to 30 min in ApoE mice, while it peaked at 3 min in BL6 mice, returning to baseline thereafter. The elevated HCA after IV injection of C activators is "paradoxical" in mice, since it implies an increase rather than a decrease in C levels in the blood. One possible explanation of the phenomenon is hemoconcentration due to anaphylatoxin-induced capillary leakage, resulting in an apparent rise of HCA. In conclusion, these preliminary observations highlight, for the first time, a species-dependent opposing impact of C activation and the resulting anaphylatoxin actions on hemolytic complement activity.

Keywords: anaphylatoxins; anaphylaxis; ApoE; complement; hypersensitivity reactions.

Introduction

Complement (C) activation in blood leads to characteristic immunological, cardiopulmonary and hematological changes, which are also observed in patients undergoing cardiac surgery (1), as well as following treatments with nanomedicines (2, 3). Hence, the hypersensitivity reactions (HSRs) caused by nanomedicines are referred to as C activation-related pseudoallergy (CARPA). These symptoms can also be induced in animals by i.v. injection of zymosan, a well-known C activator (4–6), and the liposomal drugs AmBisome and Caelyx, which are state-of-art i.v. medicines against systemic fungal infection and cancer, respectively (5–8).

With the ultimate goal of establishing a mouse model of CARPA, the present study explored the effects of zymosan, AmBisome and Caelyx on hemolytic C activity (HCA) of the blood in normal C57BL/6 (BL6) and apolipoprotein E-deficient mice on BL6 background (ApoE). The latter strain was used on the basis that ApoE mice were reported to display enhanced sensitivity to C5a anaphylatoxin effects because of increased expression of the C5a receptor CD88 in macrophages, smooth muscle and activated endothelial cells (9, 10). We report here our preliminary findings suggesting the feasibility of using these mouse strains as a CARPA model, with HCA measured by the sheep red blood cell (SRBC) hemolysis assay. However, when measuring C activation the change is an elevation of plasma HCA, not a decrease, as expected from C consumption. The study provides a hypothesis regarding the mechanism of this paradoxical "hypercomplementemia".

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Materials and methods

Animals and experimental protocol

Male BL6 and ApoE mice (24–28 weeks old weighing 25–35 g; a total of 70 animals) were used in these studies. All procedures were performed in accordance with guidelines set by the National Institutes of Health (USA), and the Hungarian law on animal care and protection. All experimental protocols were approved by the Institutional Ethical Committee for Animal Care and Use of Semmelweis University.

The animals were anesthetized by isoflurane and were injected i.v. (via tail vein) with zymosan, liposomes and saline, as specified in the text. One minute before the proper time for blood sampling (3–4 mice at each time point), mice were injected with heparin (500 IU/mL) intraperitoneally, and then euthanized using isoflurane. At time 0 mice were treated with saline instead of zymosan or liposomes and these results were used as control for normalization. According to our previous experience injection of saline caused no change in HCA of the plasma over time. Blood was collected from the transected caval vein.

Hemolytic C assay

A modified hemolytic C (CH50) assay was used in our study. Fresh SRBCs were obtained from a local vendor, and were washed and suspended in Dextrose-Gelatin-Veronal buffer (DGV; Lonza Verviers, Belgium). Cells were sensitized with anti-sheep RBC antibody (1:500 vol/vol) at 37°C for 30 min, and then washed twice with iced DGV. Mouse plasma samples taken at the indicated times were supplemented with C3 depleted human serum (TECOmedical AG, Sissach, Switzerland) followed by a 10-fold dilution in PBS and incubated with sensitized SRBCs for 10 min at 37°C. The sample volume of the 10-fold diluted plasma was adjusted so that the degree of hemolysis fell on the steep part of the hemolysis vs. volume sigmoid curve. The reaction was stopped by centrifugation of the cells (at 4°C, 400×*g* for 4 min) and the released hemoglobin in the supernatant was measured at 541 nm using 96-well plates and FLUOstar Omega Plate Reader (BMG Labtech, Ortenberg, Germany). Distilled water was used as positive, and phosphate-buffered saline (PBS) as negative control. All measurements were performed in duplicates.

Results

Figure 1 shows the changes in HCA in normal BL6 (A) and ApoE (B) mice following i.v. injection of 0.5 mg/kg zymosan. In both cases the HCA started to rise immediately following the injections, however, in normal mice the rise reached its maximum at 3 min and the subsequent readings indicated a decline to baseline level (A), while in ApoE mice the rise of HCA was steady over 30 min and reached statistical significance ($p<0.05$) at 30 min (B).

To reproduce and extend the above preliminary data, a second set of experiments were performed with samplings only at 0 (saline control), 5 and 15 min, using a 10-fold higher dose of zymosan. In this shortened experiment, we also tested AmBisome (10 mg/kg, phospholipid: 2.2 mg/kg) and Caelyx (16 mg/kg, phospholipid: 12.8 mg/kg), i.e. C activating, CARPA-genic liposomes.

As shown in Table 1, in normal BL6 mice zymosan and AmBisome caused massive, significant rises of HCA at 15 min, also done byzymosan at 5 min, thus confirming the observations in Figure 1. In ApoE-deficient mice both liposomes caused significant rises of HCA at 15 min, while zymosan tended to increase HCA at 5 min, although this difference was not significant. Importantly, however, this high dose of zymosan led to a decreased HCA in ApoE mice at 15 min. Taken together, these data suggest a complex picture where different C activators have varying effects on C consumption and, assumedly, capillary permeability, in normal and C5a sensitized mice, and the actual HCA depends on a fine balance between these opposing processes. In any case, the occurrence of a paradoxical rise of HCA was again present in this second experiment.

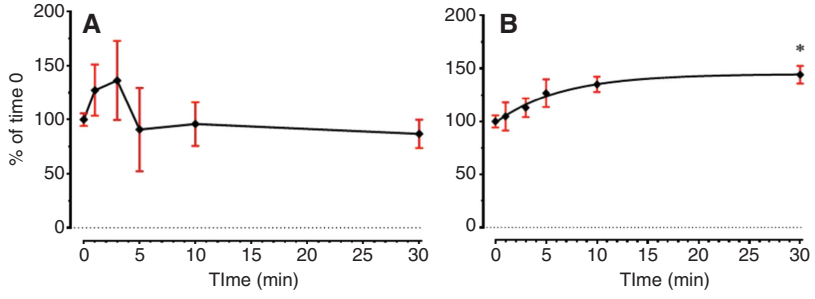

Figure 1: Effect of zymosan (0.5 mg/kg) on hemolytic complement activity (HCA) in BL6 (A) and ApoE (B) mice. The data are expressed as hemoglobin OD_{541}, % of saline control (0 min), mean±SD for n=3–4 mice at each time point. The 0 min value (OD_{541}: 0.51±0.13, n=4) represents immediate sampling after injection of saline at a volume (50 µL/10 g) that corresponds to the volume of zymosan and liposome administrations. HCA was determined by the SRBC assay, as described in the methods. For statistical analysis one way ANOVA was used. *$p<0.05$.

Table 1: Effect of zymosan, Ambisome and Caelyx on hemolytic C activity (HCA) in BL6 and ApoE mice. Entries are mean±SD (n=3–4) hemoglobin OD_{541} related to 0 min saline baseline (%).

	Saline	Zymosan (5 mg/kg)		AmBisome (2.2 mg PL/kg)		Caelyx (12.8 mg PL/kg)
	0 min	5 min	15 min	5 min	15 min	15 min
BL6	100	153.1±34.2[a]	152.9±24.4[a]	NA	171.1±12.6[a]	NA
ApoE	100	111.8±21.8	84.3±11.8	101.3±4.8	116.5±2.6[b]	114.3±2.4[b]

[a]$p<0.05$. [b]$p<0.01$ relative to saline control. For statistical analysis one-way ANOVA and Dunnett's multiple comparison test was used. Liposome doses are given as phospholipid (PL) content. NA, not administered.

Discussion

CARPA is a C-mediated HSR that frequently occurs in humans following i.v. administration of nanomedicines, including AmBisome and Caelyx (2, 3, 8, 11). Because of its unpredictability and occasional lethality, the reaction represents a safety issue in the R&D of liposomal and other i.v. drugs (8, 11), whose analysis was recently recommended by the European Medicines Agency to confirm the lack of risk of C-mediated anaphylaxis (12). However, at this time there is no consensus regarding the question, which C and CARPA assays should be used under what conditions, and how. The assessment of C activation and CARPA caused by different nanomedicines have not been standardized or regulated to date, presenting a perplexing variety of assay options for those who intend to explore the immune compatibility of their nanomedicinal drug candidates.

The ultimate goal of the present study was to address this question and to develop an in vivo assay in mice for CARPA. Earlier we developed a porcine model, which is highly sensitive and mimics the reactions seen in hypersensitive men (5, 13, 14), however, this model requires special facility, equipment and skilled personnel. The alternative rat model also produces many of the typical CARPA symptoms, but rats are 2–3 orders of magnitude less sensitive to CARPA than pigs are (14). So, in the present study we turned our attention to mice, as a possible CARPA model.

As for previous evidence of CARPA in mice, Wang et al. investigated C activation in mice by the highly CARPAgenic Taxol and a liposome-based alternative paclitaxel formulation (15). They showed significant rise of serum SC5b-9 following treatment with Taxol and less rise with the liposome formulation, suggesting that CARPA in fact occurs and can be tested in mice. However, to avoid the use of the SC5b-9 ELISA, which was questioned in other studies (16, 17), we made an attempt to use the simplest and least expensive C test, the classical SRBC assay to measure C activation in mice. Our experiments validate the use of a simplified version of this assay.

As for the test agents used for C activation, zymosan has been known as the gold standard to induce C activation in vitro as well as in vivo. It is a glycoside component of the cell wall of yeasts (e.g. *Saccharomyces cerevisiae*), a glucan structure with repeating glucose units connected by β-1,3-glycosidic linkages. It activates nuclear factor-κB (NF-κB), signaling in resident macrophages via binding to Toll-like (TLR2) receptor (4). Intravenous treatment with zymosan reduces serum C hemolytic activity and causes all known symptoms of CARPA (leukopenia, thrombocytopenia, decreased blood pressure and increased hematocrit, edema) in different species, including pigs and rats (7, 13, 14, 18, 19). The exact mechanism of these changes is complex and likely to involve direct macrophage and other cellular effects in addition to C activation, but C activation is definitely a major contributing factor, rationalizing its use in the mouse model. Regarding its dose, in the pig model 0.1–0.5 mg/kg zymosan was found to be sufficient to elicit cardiovascular reactions (13, 20), whereas the effective dose for similar effects was 1–10 mg/kg in rats (14, 18). These values guided our testing of 0.5 and 5 mg/kg in the present studies. Likewise, the applied liposome doses had literature precedents (5, 14).

The choice of ApoE mice for the CARPA model was based on the information that these mice overexpress the C5a receptor (CD88) in their macrophages, smooth muscle cells and activated endothelial cells, i.e. cells that play key roles in C5a-induced anaphylaxis. It was expected that the signs of anaphylaxis would be more pronounced in these animals, and in fact, we found differences between ApoE-deficient mice compared to their wild type BL6 control in their responses to zymosan and liposomes. Most clearly, 0.5 mg/kg zymosan caused a steady rise of HCA over 30 min in ApoE mice, while it caused only a transient early rise in BL6 animals.

In summary, the present pilot study achieved its goal as we found that mice, particularly ApoE mice, did display quantifiable changes in the simplest SRBC assay. However, unexpectedly, our data suggest that it is not C

depletion (decline of HCA) that serves as a quantitative end-point for our CARPA assay, but rather a rise in HCA. At present we try to explain this paradoxical rise of HCA by hemoconcentration, which arises as a consequence of anaphylatoxin-triggered increase in capillary permeability. The latter phenomenon is widely known in the literature for a variety of C activation situations, including those induced by zymosan. We are aware that on the basis of a single measure, i.e. HCA, it is premature to conclude to hemoconcentration as the definite cause of HCA rise, and emphasize that it is only a hypothesis at this moment. It needs to be confirmed by a broad range of additional, complementary evidence, like the rise of hematocrit, direct evidence of capillary leakage, establishment of the molecular mechanism of C activation-related capillary leakage, which was previously associated with histamine (21) and leukotrienes (22). Such studies are underway in our laboratory. However, the main message of the present rapid communication is the paradoxical rise of HCA, which was reproduced in two mouse strains, two series of experiments and three C activators, providing confidence for its communication in the present state of research. To our best knowledge, the observation is novel in the literature. In addition, we serendipitously identified a KO mouse strain that might be sensitized for the phenomenon, which is explainable by the underlying gene defect (overexpression of C5a receptors in the capillary smooth muscle and endothelial cells). Clearly, further studies are needed to circumnavigate and further understand the phenomenon of paradoxical "hypercomplementemia" as a novel endpoint of CARPA in mice.

Conclusions

Intravenous treatment with zymosan and liposomal drugs (AmBisome, Caelyx) led to a significant rise in hemolytic complement activity of the plasma in BL6 and ApoE mice in striking contrast to observations made in pigs, dogs, rats and other species, in which administration of the C activators decreases hemolytic complement activity. The paradoxical reaction of mice can most easily be explained by hemoconcentration due to anaphylatoxin-induced capillary leakage resulting in an apparent rise of hemolytic complement activity of the plasma.

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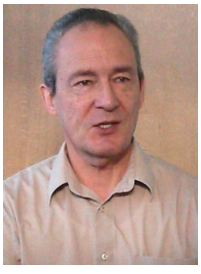

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