Chapter 13

Analysis of Complement Activation by Nanoparticles

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Abstract

The complement system is a group of proteins, which function in plasma to assist the innate immunity in rapid clearance of pathogens. The complement system also contributes to coordination of the adaptive immune response. Complement Activation Related Pseudo Allergy or CARPA is a life-threatening condition commonly reported with certain types of drugs and nanotechnology-based combination products. While CARPA symptoms are similar to that of anaphylaxis, the mechanism behind this pathology does not involve IgE and is mediated by the complement system. In vitro assays using serum or plasma derived from healthy donor volunteers correlate with the in vivo complement-mediated reactions, and therefore are helpful in understanding the propensity of a given drug formulation to cause CARPA in patients. In the first edition of this book, we have described an in vitro method for qualitative assessment of the complement activation by nanomaterials using western blotting. Herein, we present a similar method utilizing enzyme-linked immunoassay for quantitative analysis of the complement activation, and we compare the performance of this approach to that of the qualitative western blotting technique. The revised chapter also includes new details about nanoparticle sample preparation.

Key words Nanoparticles, Complement, Anaphylaxis, C3, Western blot, Immunoassay, EIA

1 Introduction

The complement system is comprised of several components organized into a biochemical cascade serving to assist the immune system in the clearance of pathogens [1]. The biochemical cascade includes three main pathways—classical, alternative, and lectin (Fig. 1). Activation of each of these pathways is triggered by different factors. For example, an immune complex composed of an antibody and an antigen is required to activate the classical pathway. Activation of the alternative pathway does not oblige an antibody and depends on spontaneous hydrolysis of a C3 component of the complement and properdin. Mannose-binding lectin triggers activation of the mannose pathway. These pathways converge on C3 component [1]. Cleavage of the C3 protein results in activation of the common pathway that culminates with formation of the terminal or membrane attack complex. Complement cleavage products

Scott E. McNeil (ed.), *Characterization of Nanoparticles Intended for Drug Delivery*, Methods in Molecular Biology, Vol. 1682, DOI 10.1007/978-1-4939-7352-1_13,

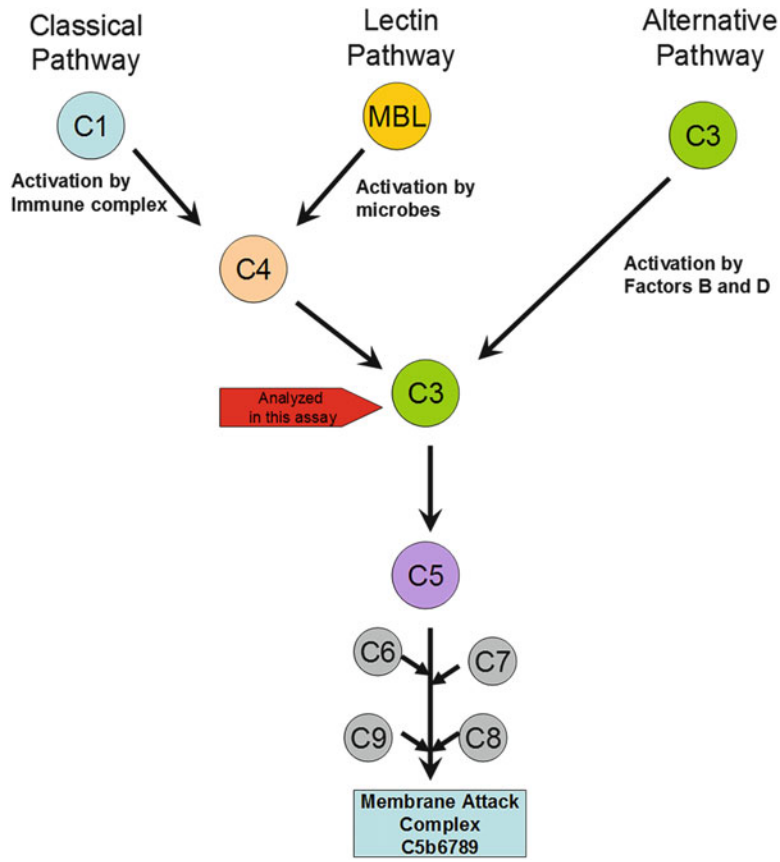

Fig. 1 Complement activation pathways. The antibodies used in this assay detect split product of C3 component of complement

C4a, C3a, and C5a are potent immunostimulants also known as anaphylatoxins. The presence of these proteins in the bloodstream is associated with induction of inflammation, generation of oxygen radicals, increase in vascular permeability, the IgE-independent release of histamine from mast cells, and smooth muscle contraction [1]. The complement terminal complex functions to destroy the pathogen, and in the absence of such (e.g., during drug-mediated complement activation) it damages healthy host cells [2–5]. Altogether these responses produce anaphylaxis and tissue damage. Since complement activation enhances antigen presentation, a certain degree of complement activation is desirable for vaccines [6]. However, for systemically administered drugs and combination products, the complement activation is undesirable due to the CARPA reaction [2–5, 7–12].

Complement-mediated infusion reactions and CARPA are common dose-limiting toxicities for particular types of drug products including therapeutic oligonucleotides [9–11] and PEGylated liposomal formulations of small molecules [4, 5, 12].

For example, liposomal formulations of doxorubicin (Doxil) and amphotericin (Ambisome), when administered into systemic circulation, activate the complement and result in complement-mediated hypersensitivity reactions in sensitive individuals [3, 5, 7, 8, 12]. In contrast, subcutaneously administered polymer-based nanoparticles, which lead to local complement activation, improve vaccine efficacy by enhancing antigen uptake by dendritic cells, activating T cells and supplementing the antigen-specific immune response [6].

Nanoparticle physicochemical properties, including size, charge, shape, and surface functionalities, determine the particle interaction with the complement system. For example, charged nanoparticles were shown to be more potent activators of the complement system than their neutral counterparts in studies investigating polypropylene sulfide nanoparticles, lipid nanocapsules, cyclodextrin-containing polycation-based nanoparticles, and polystyrene nanospheres [13–18]. Polymer coatings (such as polyethylene glycol (PEG) and poloxamine 908), which partially neutralize surface charge, have been shown to reduce nanoparticle-mediated complement activation [13, 16]. Similar studies using dextran and chitosan coatings reported that charge in combination with size and conformation of the polymer played the key role in complement activation by these particles [17]. In the case of Doxil, the combination of three factors, including particle shape, PEG coating, and the presence of doxorubicin crystals, determines complement activation [8, 19, 20]. Dr. Janos Szebeni proposed using analysis of complement activation by PEGylated liposomal drugs to assess the bioequivalence of generic formulations [3]. In vitro–in vivo correlation of complement activation is reviewed elsewhere [21].

In the first edition of this book, we described an in vitro method utilizing western blotting technique to perform qualitative analysis of the complement activation by nanoparticles [22]. Herein, we describe a similar method using quantitative analysis of the C3 component of complement split product iC3b. In this protocol, a test sample is incubated with human plasma, and the amounts of iC3b protein are measured by an enzyme-linked immunoassay (EIA). The amount of iC3b is proportional to the level of complement activation. Other split products, such as C3a, can also be used to perform this analysis. We selected iC3b because this analysis generates a higher signal-to-noise ratio (Fig. 2). The revised method includes considerations for selecting nanoparticle concentrations for in vitro analysis. We also compare the performance of qualitative western blotting and quantitative EIA methods for analysis of the complement activation by nanomaterials. This comparison demonstrates that western blotting is a more sensitive technique than EIA (Fig. 3). The greater sensitivity is explained by the use of polyclonal antibodies which capture all

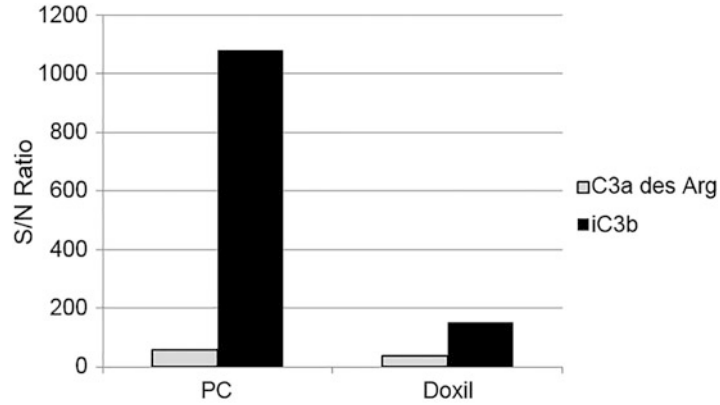

Fig. 2 Comparison between C3a and iC3b EIAs. Plasma samples treated with negative control, positive (cobra venom factor), and Doxil were analyzed by C3a des Arg and iC3b EIA. Each sample was analyzed in duplicate (%CV < 10). The mean responses from positive control (PC) and Doxil samples were divided by the mean response of the negative control sample to estimate the signal-to-noise (S/N) ratio for each EIA. The background in the C3a EIA was higher. Due to the differences in the background, the S/N ratio of iC3b assay was higher

split products. Despite the difference in sensitivities, EIA allows detection of the complement at the levels relevant to CARPA induction in vivo. The western blot detects lower levels of complement split products which may be asymptomatic in vivo. While both methods can be used for analysis of the complement activation, EIA overcomes limitations of the western blotting technique such as low throughput. Although the protocol described herein focuses on measuring iC3b component of the complement, supernatants generated in this test can be used to measure other complement components (e.g., C4a, C4d, and Bb) when identification of the pathway responsible for the complement activation by a test nanomaterial is needed.

2 Materials

1. Sterile Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS).
2. Cobra venom factor as the positive control.
3. Veronal buffer.
4. Pooled human plasma, anti-coagulated with sodium citrate.
5. iC3b EIA kit (e.g., MicroVue by Quidel Corp.).
6. 1.0 N HCl, as stop solution. Stop solution is provided with each kit, but can also be prepared separately. Dilute stock hydrochloric acid to a final concentration of 1.0 N. Filter and store and room temperature for up to 2 weeks.

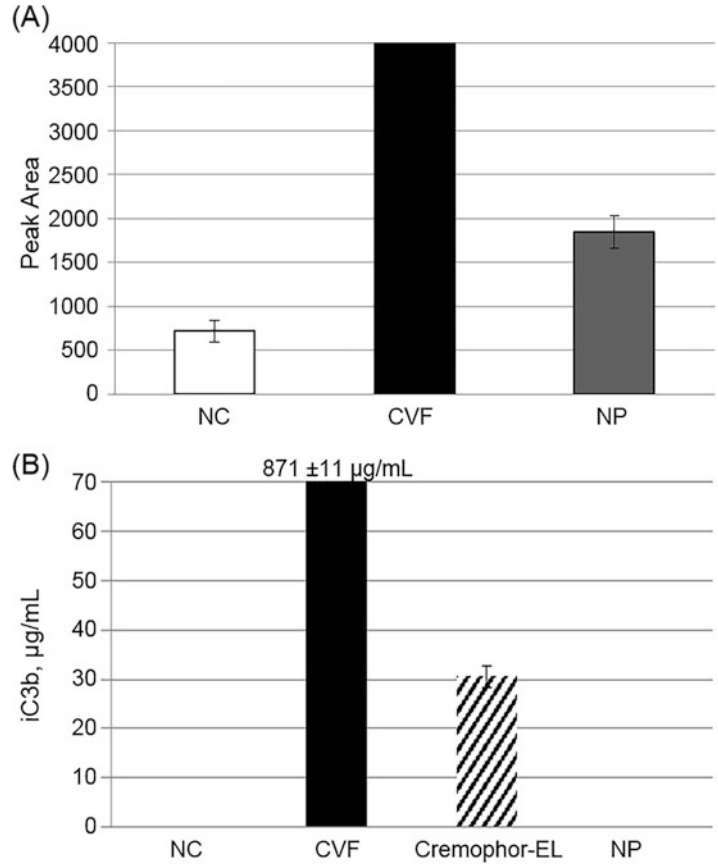

Fig. 3 Comparison between western blotting and EIA of iC3b. The samples of plasma treated with negative control, positive control, and nanoparticle were analyzed by western blot (**a**) and EIA (**b**). Each bar shows mean and SD of duplicate response. (**a**) Cremophor-EL was not analyzed in western blot assay because the presence of oil affects protein mobility and results in smeared bands. Western blot results were analyzed by the NIH ImageJ software and shown is the peak intensity of the band corresponding to C3 split products. (**b**) The scale was changed to show levels of iC3b in Cremophor-EL and nanoparticle-treated samples (NP). The level of iC3b in the positive control sample (CVF) is 871 ± 11 µg/mL. NC: negative control, NP: nanoparticle, CVF: cobra venom factor

7. Doxil (Doxorubicin HCl, liposome, injection). This is a prescription medication available from a licensed pharmacy. It may not be available to some research laboratories.
8. Cremophor.
9. Taxol (Paclitaxel in Cremophor EL). This is a prescription medication available from a licensed pharmacy. This drug may not be available to some research laboratories.
10. Multichannel (8–12 channel) pipettor for 50–300 µL volumes.

11. Reagent reservoirs.
12. ELISA plate reader capable of operating at 405 nm.
13. *See* **Note 1**.

3 Methods

3.1 Preparation of Controls and Plasma

1. Prepare Cobra Venom Factor (CVF) as Positive Control 1, a traditional substance known to activate complement. CVF is supplied as a frozen solution. Thaw this stock, prepare single-use aliquots and store them at a nominal temperature of −80 °C as long as performance is acceptable. Avoid repeated freeze/thaw cycles. After thawing single-use aliquot and using it in the assay, discard any leftover material. For this experiment, use 30 μL (1.1–50 U) of CVF solution. This control activates the complement system through the alternative pathway (*see* **Note 2**).
2. Prepare Positive Control 2, a nanoparticle-relevant control.

   Cremophor-EL is an excipient commonly used in the pharmaceutical industry to dissolve hydrophobic drugs. Cremophor-EL is a nanosized micelle, which is known to induce CARPA syndrome [5], and therefore is used as a nanoparticle-relevant control. The following procedure can be used to prepare Cremophor-EL with the composition similar to that in clinical formulation of paclitaxel (Taxol): 527 mg of purified Cremophor® EL* (polyoxyethylated castor oil) and 49.7% (v/v) dehydrated alcohol, USP and 2 mg of citric acid per 1 mL. Store at room temperature. To prepare Cremophor-EL mix commercial Cremophor 1:1 with ethanol containing 2 mg/mL of citric acid to mimic the concentration of Cremophor-EL, citric acid, and ethanol used in Taxol® and the generic formulation of paclitaxel.

   Cremophor-EL-formulated Paclitaxel (Taxol) can be used as an alternative for this nanoparticle-relevant positive control. It is supplied at a stock concentration of 6 mg/mL of paclitaxel. When used in this assay, the final concentration of Paclitaxel is 2 mg/mL. Store at 2–8 °C.

   PEGylated liposomal doxorubicin (Doxil) can also be used as a nanoparticle-relevant positive control [3]. Doxil is doxorubicin formulated in nanoliposomes. It is available through the pharmacy as 20 mg of Doxorubicin HCl in 10 mL of vehicle. Store at 2–8 °C.
3. Prepare the Inhibition/Enhancement Control. Use the positive control sample after incubation. Prior to loading this sample onto the ELISA plate, add nanoparticles at the same final concentrations as in the study samples. For example, one can

mix 20 μL of the positive control sample and 10 μL of the test nanoparticle. The test result for this sample needs to be adjusted by the dilution factor 1.5 prior to comparison to the test value of the positive control sample. If the test results do not differ more than 25% of each other, the test nanoparticle at the given concentration does not interfere with the detection of the complement split product by ELISA.

4. Prepare the Negative Control. Sterile Ca^{2+}/Mg^{2+}-free PBS is used as a negative control. Store at room temperature for up to 6 months.
5. Prepare the Vehicle Control, which is relevant to the given nanoparticle. When nanoparticles are formulated in a vehicle other than saline or PBS, the vehicle sample should be tested to estimate the effect of excipients on the complement system. This control is specific to each given nanoparticle sample. It should be prepared to match the formulation buffer of the nanoparticle by both the composition and the concentration.
6. To prepare plasma, the blood is spun down in a centrifuge for 10 min at 2500 × *g* (*see* **Note 3**).

3.2 Preparation of Nanoparticle Samples

This assay requires 400 μL of nanoparticles in PBS at a concentration three times higher than the highest final tested concentration. The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol, this concentration is called the "theoretical plasma concentration." Considerations for estimating theoretical plasma concentration were reviewed elsewhere [21] and are also summarized in Box 1. The assay will evaluate four concentrations: 10× (or when feasible 100×, 30×. or 5×) of the theoretical plasma concentration, theoretical plasma concentration, and two serial 5-fold dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration. For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 6 mg/mL will be prepared and diluted 10-fold (0.6 mg/mL), followed by two serial 5-fold dilutions (0.12 and 0.024 mg/mL). When 0.1 mL of each of these samples is added to the test tube and mixed with 0.1 mL of plasma and 0.1 mL of veronal buffer, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04, and 0.008 mg/mL.

3.3 Assay

1. In a microcentrifuge tube, combine equal volumes (100 μL of each) of veronal buffer, human plasma, and a test sample (i.e., positive control, negative control, nanoparticles, or vehicle control if different than PBS). Prepare two replicates of each sample.

Box 1. Example Calculation of Nanoparticle Concentration for In Vitro Test

Assume the mouse dose is known to be 123 mg/kg.

$$\text{human dose} = \frac{mouse\ dose}{12.3} = \frac{123\frac{mg}{kg}}{12.3} = 10\ \text{mg/kg}$$

Blood volume constitutes approximately 8% of body weight (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows for a very rough estimation of what the maximum blood concentration may be.

$$\text{in vitro concentration}_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg\ x\ 10\frac{mg}{kg}}{5.6\ L}$$

$$= \frac{700\ mg}{5.6\ L} = 0.125\ \text{mg/mL}$$

Box 1 Example calculation of nanoparticle concentrations for in vitro tests. Reproduced with permission from ref. 21

2. Vortex tubes to mix all reaction components, spin briefly in a microcentrifuge to bring any drops down, and incubate in an incubator at a nominal temperature of 37 °C for 30 min.
3. Prepare 100 μL aliquots and either use in EIA immediately or freeze at −20 °C for later analysis.
4. Follow the manufacturer's instruction to reconstitute complement standard, buffers, and controls.
5. Dilute plasma samples prepared in **step 3** in complement specimen diluent reagent (provided with each kit). Use the following dilution guide for each individual assay (*see* **Note 4**):

   iC3b—1:1500 for positive control sample; 1:75 for negative control, and other test samples.
   C4d—1:30 for all samples.
   Bb—1:75 for all samples.
6. Follow the manufacturer's instruction for plate loading volumes, incubation time, and plate washing.

3.4 Calculations and Results Interpretation

Do not forget to use the appropriate dilution factor for control and study samples. Compare determined amount of complement components between positive control or study samples with that in the negative control. An increase in the complement component species two fold or higher above the background (negative control) constitutes a positive response. If a nanoparticle under study generated a positive response in any of the EIA assays, compare the degree of activation between this particle and Doxil or other nanoparticle-relevant controls. Doxil is used in the clinic and is

known to induce complement activation-related hypersensitivity reactions in sensitive patients [5]. Using Doxil helps to interpret results of this in vitro study for a test nanoparticle. If the degree of activation observed for the test nanoparticle is equal to or greater than that observed for Doxil, this nanoparticle formulation will most likely cause similar or stronger hypersensitivity reactions in patients and may require modifications before entering in vivo preclinical and clinical phases. If the degree of activation is lower than that of Doxil, complement activation should be considered when designing the in vivo evaluation phase for the given particle; but, it is less likely to cause concerns similar to Doxil.

4 Notes

1. NCL does not endorse suppliers. However, we found that a new user benefits from knowing catalog information of reagents used in our assays. If you need ideas of what reagents are used at the NCL, please review NCL method ITA-5 available at https://ncl.cancer.gov/resources/assay-cascade-protocols. When other reagents are used, the assay performance may change. When using reagents and instruments from sources other than that used in our protocols, assay performance qualification is needed to verify the assay functionality and validity.
2. *Heat Aggregated Gamma Globulin (HAGG)* acts similarly to naturally occurring immune complexes and is a very potent activator of complement through the classical pathway. HAGG can be used as Positive Control 1 when activation of the complement through the classical pathway is desired. This control is available from Quidel under the name "Complement Activator." Handling and storage are according to the manufacturer's instructions. Avoid repeated freeze/thaw cycles when stored at −20 °C.
3. Blood is drawn into vacutainer tubes containing anticoagulant. Sodium citrate is an ideal anticoagulant for this assay. However, depending on phlebotomy paraphernalia, plasma anticoagulated with sodium citrate may result in a high background in the ELISA assay. In this case, using K2 EDTA as an anticoagulant is acceptable. The first 5–10 mL of blood should be discarded and not used to prepare plasma. For optimal results, it is important to keep blood at 20–24 °C to avoid exposure to high temperatures (summer time) and low temperatures (winter time), and to avoid prolonged (> 1 h) storage. Blood is transported to the lab in a contained Styrofoam box with warm packs (20–24 °C). After centrifugation to separate plasma, the plasma is evaluated for the presence of hemolysis. Discolored

plasma (an indication of hemolysis) is not used to prepare the pool. Individual plasma specimens that did not show any indication of hemolysis are pooled and mixed in a conical tube. Plasma must be used for complement testing within 1 h after collection. Pooled plasma can be used and prepared by mixing plasma from at least 2 individual donors. The assay can also be performed in plasma from individual donors. In this case, analyze plasma from at least 3 donors. Inter-individual variability and comparison to pooled plasma is shown in Fig. 4.

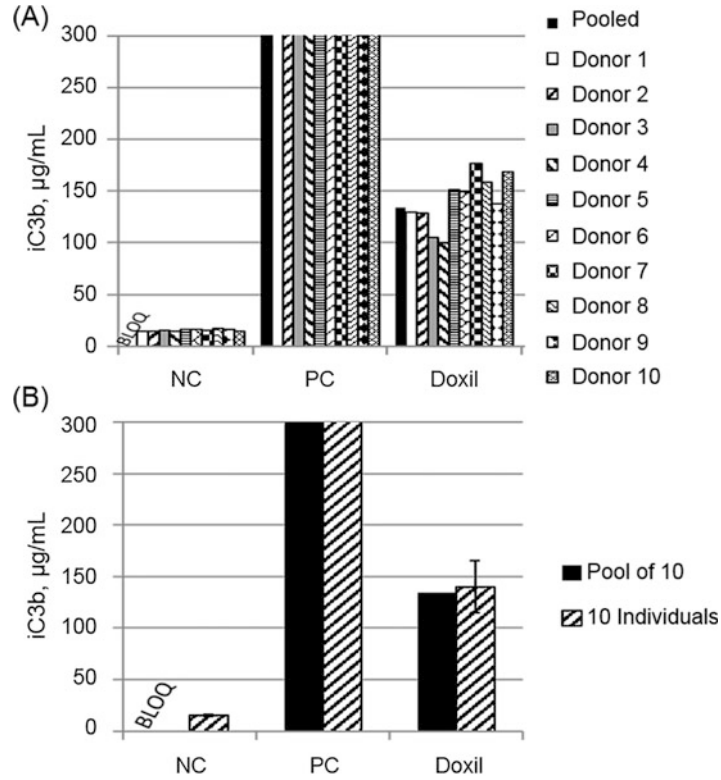

Fig. 4 Analysis of complement activation by Doxil in individual and pooled plasma. Plasma from ten donors was used to analyze Doxil. The plasma was tested either individually or after pooling. To prepare pooled plasma equal volumes of individual plasmas were mixed. The analysis was performed according to the experimental procedure described in this chapter. (**a**) Shows results for controls and Doxil in individual plasma from 10 donors. Each bar represents a mean and SD from 2 replicates, %CV between replicates is less than 10. (**b**) Compares result generated in plasma pooled from 10 donors (*black bar*, pooled plasma) and the mean result of ten individual responses analyzed side-by-side (*hatched bars*, ten individuals). *Black bar* shows a mean and SD from two replicates, %CV between replicates is less than 10. Hatched bars show the mean result and SD ($N = 10$)

It is possible to use pooled sodium citrate plasma from commercial suppliers; however, when placing the order, one needs to notify the supplier that the plasma is intended for complement testing so no delays between blood draw and plasma collection occur. The supplier then freezes the plasma immediately after collection and ships it to the lab on dry ice. When using frozen plasma for the complement activation assay, it is important to avoid repeated freeze/thaw cycles. The frozen plasma should be thawed in a water bath containing ambient tap water, mixed gently, and used immediately after thawing. It is also advised to avoid indefinite storage of frozen plasma at −20 °C. The sooner the frozen plasma is used, the better the results are. In general, the degree of complement activation estimated by comparing intensity of the C3 split product in the positive control with that of the negative control is greater in fresh plasma than in thawed plasma.

4. The dilution factors should be determined by each laboratory and adjusted as needed.

Acknowledgment

This project has been funded in whole or in part by federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

1. Calame DG, Mueller-Ortiz SL, Wetsel RA (2016) Innate and adaptive immunologic functions of complement in the host response to Listeria monocytogenes infection. Immunobiology 221(12):1407–1417. doi:10.1016/j.imbio.2016.07.004
2. Szebeni J, Fishbane S, Hedenus M, Howaldt S, Locatelli F, Patni S, Rampton D, Weiss G, Folkersen J (2015) Hypersensitivity to intravenous iron: classification, terminology, mechanisms and management. Br J Pharmacol 172 (21):5025–5036. doi:10.1111/bph.13268
3. Szebeni J, Storm G (2015) Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs. Biochem Biophys Res Commun 468(3):490–497. doi:10.1016/j.bbrc.2015.06.177
4. Rampton D, Folkersen J, Fishbane S, Hedenus M, Howaldt S, Locatelli F, Patni S, Szebeni J, Weiss G (2014) Hypersensitivity reactions to intravenous iron: guidance for risk minimization and management. Haematologica 99 (11):1671–1676. doi:10.3324/haematol.2014.111492
5. Szebeni J (2014) Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. Mol Immunol 61(2):163–173. doi:10.1016/j.molimm.2014.06.038
6. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, Lee LK, Swartz

MA, Hubbell JA (2007) Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol 25 (10):1159–1164. doi:10.1038/nbt1332

7. Moghimi SM, Wibroe PP, Szebeni J, Hunter AC (2013) Surfactant-mediated complement activation in beagle dogs. Int Immunopharmacol 17(1):33–34. doi:10.1016/j.intimp.2013.05.012
8. Szebeni J, Muggia F, Gabizon A, Barenholz Y (2011) Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention. Adv Drug Deliv Rev 63 (12):1020–1030. doi:10.1016/j.addr.2011.06.017
9. Shen L, Engelhardt JA, Hung G, Yee J, Kikkawa R, Matson J, Tayefeh B, Machemer T, Giclas PC, Henry SP (2016) Effects of repeated complement activation associated with chronic treatment of Cynomolgus monkeys with 2′-O-Methoxyethyl modified antisense oligonucleotide. Nucleic Acid Ther 26(4):236–249. doi:10.1089/nat.2015.0584
10. Shen L, Frazer-Abel A, Reynolds PR, Giclas PC, Chappell A, Pangburn MK, Younis H, Henry SP (2014) Mechanistic understanding for the greater sensitivity of monkeys to antisense oligonucleotide-mediated complement activation compared with humans. J Pharmacol Exp Ther 351(3):709–717. doi:10.1124/jpet.114.219378
11. Henry SP, Beattie G, Yeh G, Chappel A, Giclas P, Mortari A, Jagels MA, Kornbrust DJ, Levin AA (2002) Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. Int Immunopharmacol 2(12):1657–1666
12. Chanan-Khan A, Szebeni J, Savay S, Liebes L, Rafique NM, Alving CR, Muggia FM (2003) Complement activation following first exposure to pegylated liposomal doxorubicin (Doxil®): possible role in hypersensitivity reactions. Ann Oncol 14(9):1430–1437
13. Vonarbourg A, Passirani C, Saulnier P, Simard P, Leroux JC, Benoit JP (2006) Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake. J Biomed Mater Res A 78 (3):620–628. doi:10.1002/jbm.a.30711
14. Bartlett DW, Davis ME (2007) Physicochemical and biological characterization of targeted, nucleic acid-containing nanoparticles. Bioconjug Chem 18(2):456–468. doi:10.1021/bc0603539
15. Nagayama S, Ogawara K, Fukuoka Y, Higaki K, Kimura T (2007) Time-dependent changes in opsonin amount associated on nanoparticles alter their hepatic uptake characteristics. Int J Pharm 342(1–2):215–221. doi:10.1016/j.ijpharm.2007.04.036
16. Al-Hanbali O, Rutt KJ, Sarker DK, Hunter AC, Moghimi SM (2006) Concentration dependent structural ordering of poloxamine 908 on polystyrene nanoparticles and their modulatory role on complement consumption. J Nanosci Nanotechnol 6(9–10):3126–3133
17. Bertholon I, Vauthier C, Labarre D (2006) Complement activation by core-shell poly(isobutylcyanoacrylate)-polysaccharide nanoparticles: influences of surface morphology, length, and type of polysaccharide. Pharm Res 23 (6):1313–1323. doi:10.1007/s11095-006-0069-0
18. Xu Y, Ma M, Ippolito GC, Schroeder HW Jr, Carroll MC, Volanakis JE (2001) Complement activation in factor D-deficient mice. Proc Natl Acad Sci U S A 98(25):14577–14582. doi:10.1073/pnas.261428398
19. Szebeni J, Bedocs P, Rozsnyay Z, Weiszhar Z, Urbanics R, Rosivall L, Cohen R, Garbuzenko O, Bathori G, Toth M, Bunger R, Barenholz Y (2012) Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome. Nanomedicine 8 (2):176–184. doi:10.1016/j.nano.2011.06.003
20. Szebeni J, Baranyi L, Savay S, Milosevits J, Bunger R, Laverman P, Metselaar JM, Storm G, Chanan-Khan A, Liebes L, Muggia FM, Cohen R, Barenholz Y, Alving CR (2002) Role of complement activation in hypersensitivity reactions to doxil and hynic PEG liposomes: experimental and clinical studies. J Liposome Res 12(1–2):165–172. doi:10.1081/lpr-120004790
21. Dobrovolskaia MA, McNeil SE (2013) Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release 172(2):456–466. doi:10.1016/j.jconrel.2013.05.025
22. Neun BW, Dobrovolskaia MA (2011) Qualitative analysis of total complement activation by nanoparticles. Methods Mol Biol 697:237–245. doi:10.1007/978-1-60327-198-1_25