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Review

An international serum standard for application in assays to detect human complement activation products

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ABSTRACT

The importance of the complement system in clinical medicine has become evident during the last decades and complement therapeutics has now reached the clinic. Thus, there is an increased interest in and need for assays to evaluate complement activity and dysfunction. Pathologically increased complement activation can indirectly be evaluated by quantification of complement components, but in order to exactly measure such activation, assays for quantification of products formed during activation are required. Progress in this field is hampered by lack of standardization. Therefore, members of the International Complement Standardization Committee, a joint initiative of the International Complement Society and the International Union of Immunological Societies (IUIS), prepared a defined standard for application in assays for complement activation products. We here report on the production and properties of this International Complement Standard #2 (ICS#2). ICS#2 was made from a pool of sera from healthy blood donors (ICS#1) that was activated with a combination of heat-aggregated IgG and zymosan, and subsequently stabilized by adding EDTA and nafamostat mesylate. The protocol was optimized to make the standard applicable in the following activation product assays: C1rs-C1-inhibitor complexes, C4a, C4bc, C4d, Bb, C3bBbP, C3a, C3bc, C3dg, C5a and the soluble terminal C5b-9 complement complex (SC5b-9, TCC). ICS#2 was defined as containing 1000 complement activation units (CAU)/mL for all activation products measured. All activation products were stable after 10 times thawing and freezing and most of the activation products were stable during storage at 4 °C for up to 21 days. ICS#2 was produced large-scale and is considered a valuable tool for standardization, calibration and reference control for complement activation assays, providing the necessary prerequisite for quality assessments between complement laboratories.

1. Introduction

1.1. Background

Along with the increasing understanding of complement as an important part of etiology and pathophysiology of a number of disease conditions, the interest for complement has increased and the first specific complement inhibitor (eculizumab) is already in clinical use, FDA approved to treat paroxysmal nocturnal hemoglobinuria and atypical hemolytic-uremic syndrome. To further explore the role of complement in human diseases, complement laboratories must expand their assay repertoire for evaluating complement pathophysiology. Several recent reviews on complement analysis in research and clinical practice have been published and are here referred to for background information (Mollnes and Kirschfink, 2006; Mollnes et al., 2007; Oppermann and Wurzner, 2010; Harboe et al., 2011; Nilsson and Ekdahl, 2012).

Among assays to measure complement activity and function, only a few are standardized and can be compared between laboratories worldwide. A wide range of assays, both commercial and in-house made, are used to measure different complement activation products. Many of these assays are not standardized, making it difficult to compare results between different laboratories. In order to meet these challenges, the International Complement Society as well as the International Union of Immunological Societies (IUIS)

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established an International Complement Standardization Committee. Members of this committee have previously prepared and evaluated a serum standard for quantitative and functional complement analysis in normal (non-activated) human serum. This standard was named "Standard 1" (ICS#1). An activated standard was prepared for assays to quantify complement activation products, "Standard 2" (ICS#2), which we present in this paper.

1.2. Aim of the study

We aimed to obtain a uniform activated human serum standard containing defined and stable amounts of all relevant complement activation products. For this purpose, optimal conditions to prepare a single preparation suitable for classical, lectin and alternative pathway activation were defined and characterized with respect to stability in a number of assays reflecting complement activation generated in all complement pathways. The final International Complement Standard 2 (ICS#2) was then prepared on a larger scale.

2. Materials and methods

2.1. Reagents

Zymosan A (from *Saccharomyces cerevisiae*), 2,2′-azino-di(3-ethylbenzthiosoline sulfonate) (ABTS), ethylenediaminetetraacetic acid (EDTA), polyethylene sorbitan monolaurate (Tween 20) and phosphate-buffered saline (PBS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). $Na_2CO_3{\cdot}H_2O$, $NaHCO_3$, KH_2PO_4, Na_2HPO_4, NaCl and $NaC_2H_3O_2$ were obtained from Merck (Darmstadt, Germany). Gammanorm® (human IgG, 165 mg/mL) was obtained from Octapharma AB, Stockholm, Sweeden. Nafamostat mesylate (Futhan, FUT-175) was obtained from Torii Pharmaceutical Co. (Tokyo, Japan), and blood collection equipment, Vacuette®, from Greiner Bio-One International AG (Kremsmünster, Austria).

2.2. Assays for complement activation products

Three different laboratories performed a total of 12 complement activation product assays. Five in-house enzyme-linked immunosorbent assays (ELISA) were performed in Bodø, Norway, one nephelometric assay in Uppsala, Sweden, and six commercial ELISAs in Denver, Colorado.

2.2.1. In-house ELISAs

The following assays were used principally as previously described: C1rs-C1-inhibitor complexes (C1rs-C1-INH) (Fure et al., 1997), C4bc (Wolbink et al., 1993), C3bBbP (Mollnes et al., 2002), C3bc (Garred et al., 1988), and the fluid-phase terminal C5b-9 complement complex (TCC) (Mollnes et al., 1985, 1993). The assays were, however, re-established using the new ICS#2. The new detailed procedure for each assay is given in Table 1. Common for all five assays are: (1) performed using Nunc Maxisorp F96 (Nunc, Roskilde, Denmark) or Costar 3590 (Fisher Scientific, Hampton, NH). (2) Volume in all incubation steps: 100 μL/well. (3) Coat first antibodies at 4 °C for >12 h. (4) Wash 3× between each incubation in PBS with 0.1% Tween 20 (washing buffer). (5) Read plate at 405 nm with reference filter 490 nm when OD at highest standard is >0.7.

After adapting these five assays, with the new ICS#2 used for setting the standard-curve, intra- and inter-assay coefficients of variability (CV) were calculated, and were all found to be below 15% (Table 2). Lower detection limits were established and a reference range for each assay was calculated after measuring plasma samples from 20 healthy female and 20 healthy male blood donors (Table 2). Comparing statistically (Wilcoxons test) the analytical results from female and male samples, no difference was observed for any of the activation products. Thus, a common upper reference value (corresponding to the 95th percentile) was calculated for each activation product (Table 2).

2.2.2. Nephelometric assay for C3dg

C3dg was measured using a modified method described by Bourke et al. (1982). The measurement of C3dg by rocket immunoelectrophoresis in the original method was replaced by nephelometry. In brief: 200 μL EDTA plasma was mixed with an equal volume of ice-cold 22% (w/v) PEG 6000 and incubated on ice for 60 min. After centrifugation at 4000 × *g* for 15 min the C3dg concentration in the supernatant was assessed in a nephelometer using anti-C3d (DAKO, Glostrup, Denmark). The calibrator of the assay was standardized against the international standard CRM470 by using the formula ([C3dg concentration] = [C3 concentration] × [mol. weight of C3dg]/[mol. weight of C3]).

2.2.3. Commercial ELISAs

C4a OPTEIA, C3a OPTEIA and C5a OPTEIA kits were obtained from BD-Pharmingen (San Diego, CA, USA) and C4d, Bb, and SC5b-9 (TCC) from Quidel (San Diego, CA, USA). All assays were performed according to the manufacturer's instructions. In-house controls were included for each run in addition to the controls provided by the manufacturer. Each assay's performance was validated and normal ranges established using 30–60 normal donors. Intra and inter-assay variability was between 5% and 12% for all tests.

2.3. Pilot titration experiments of activators and stabilizers

A serum pool was made by collecting blood samples from 20 healthy donors into dry vacutainers, allowing them to clot for 1 h, then centrifuged at 3220 × *g* for 15 min at 4 °C and pooled before being aliquoted. They were stored at −70 °C. Heat aggregated IgG (HAIgG) was used for optimal activation of the classical pathway and zymosan A for activating the lectin and alternative pathways. HAIgG was made by heating a solution of IgG (100 mg/mL) to 63 °C for 15 min and thereafter placed directly on crushed ice until IgG precipitated.

Activation was done in serum incubated at 37 °C for different time-points. Titration of HAIgG showed maximal activation of the classical pathway at 1 mg/mL and titration of zymosan showed maximal activation of lectin and alternative pathway at 10 mg/mL. Thus, for further experiments, a combined incubation with HAIgG and zymosan in these concentrations was used. EDTA (20 mM final concentration) and nafamostat mesylate (0.2 mg/mL serum) was found to effectively block complement activation and was used in combination as stabilizer in all further experiments.

2.4. Optimization of conditions for preparation of the standard

Based on the pilot experiments, reflecting different time-curves for the different activation product assays, the serum pool was incubated with HAIgG at 1 mg/mL and zymosan at 10 mg/mL for 1, 4 and 24 h. To check for the efficacy of EDTA (20 mM) and nafamostat mesylate (0.2 mg/mL) in inhibiting the formation of the different activation products, these stabilizers were added at time point zero in parallel series. At the end of incubation (1, 4 and 24 h) the stabilizers were added to the activated serum to block further complement activation or degradation. After activation and stabilization, the serum pool was centrifuged at 4 °C for 30 min at 20,800 × *g*, followed by sterile filtration with a low protein binding Corning Filter System (cat. no. 430767; 0.2 μm CA (cellulose acetate), Corning Incorporated, NY, USA).