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A Neoepitope-Based Enzyme Immunoassay for Quantification of C1-Inhibitor in Complex with C1r and C1s

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Monoclonal antibodies (MoAb) recognizing neoepitopes exposed on activation products of complement proteins but hidden in the native components have been used for quantification of activated complement. A previously produced and characterized mouse MoAb, recognizing a neoepitope on the human plasma protein C1-inhibitor complexed with its substrates, was used to design an enzyme immunoassay for detection of C1-inhibitor complexed with C1r and C1s. These complexes are indicators of early classical complement pathway activation. The standard was serum activated with heat aggregated IgG defined to contain 1000 arbitrary units (AU)/ml. The lower detection limit was 0.05 AU/ml corresponding to 0.005% of fully activated serum. The reliability of the assay, including day-to-day variation, was tested. Intra-assay variation coefficients were 12% for low plasma control and 13% for high plasma control (n = 12 for both). Inter-assay variation coefficients were 12% for low control (n = 6), 19% for high control (n = 6) and 15% for the normal plasma control (n = 9). A 2.5–97.5 percentile reference range (normal blood donors) was 16–33 AU/ml. Two patients with systemic lupus erythematosus had considerably elevated plasma levels of the activation product (56 and 62 AU/ml), and six patients with hereditary angioedema had normal plasma levels despite considerably reduced C1-inhibitor concentration. We conclude that the present method is sensitive and reliable for detection of early classical pathway activation and superior to previously published methods by utilizing neoepitope specificity and non-radiolabelled reagents.

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INTRODUCTION

The plasma protein C1-inhibitor is the only known inhibitor of the activated complement factors C1r and C1s, and the major inhibitor of activated Factor XI, Factor XII and kallikrein of the contact pathway of coagulation [1–3]. C1-inhibitor belongs to the superfamily of serine proteinase inhibitors, the serpins. When the complement system is activated through the classical pathway, C1-inhibitor rapidly forms a complex with activated C1r and C1s. In this reaction C1-inhibitor exposes a new antigenic determinant (neoepitope), which is hidden in the native protein.

When a neoepitope-specific MoAb is used as capture antibody in an enzyme immunoassay (EIA), only the actual activation product will bind. By this approach the amount of activation product can be quantified directly in a plasma sample without any pretreatment. After washing, no native components are left in the microtiter well. A second antibody, which is not necessarily neoepitope-specific, can then be used for detection of the bound activation product [4].

Complexes containing C1r, C1s and C1-inhibitor are found in small amounts in normal plasma. Increased amounts are found in some immunological diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, chronic urticaria and primary biliary cirrhosis [5]. Furthermore, activation products of the classical complement pathway are valuable tools in biocompatibility evaluation of artificial surfaces. Until recently the complexes have been quantified by electroimmunoassay [6], neoepitope-based radioimmunoassay [7] or non-neoepitope-based radioimmunoassay [8] and EIA [9, 10]. Our aim was to establish a sensitive and specific EIA by utilizing neoepitope
specification for the detection of early classical pathway activation.

**MATERIALS AND METHODS**

*Antibodies.* The specificity of the mouse MoAb Kok 12 has been previously described in detail [7]. It reacts with a neoepitope exposed in C1-inhibitor when complexed with a protease. Goat anti-human C1s and mouse anti-human C5 were obtained from Quidel (San Diego, CA, USA), goat anti-human C1r from Nordic Immunological Laboratories (Tilburg, the Netherlands) and peroxidase-conjugated mouse anti-goat IgG from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA).

*Preparation of standard.* Activation of a normal human serum pool (NHS) from 10 healthy blood donors, by the classical pathway, was made by adding heat aggregated IgG (HAIGG). Ten mg/ml solution of NHS from 10 healthy blood donors, by the classical pathway, was incubated in a waterbath at 63 °C for 15 min. The resulting HAIGG preparation was cooled immediately, and stored at −20 °C. One mg of HAIGG per ml prewarmed serumpool was incubated for 30 min at 37 °C and then centrifuged at 6000 × g for 30 min. The supernatant was removed and stored in small aliquots at −70 °C and used as a standard in the assay.

*Samples and controls.* For handling and storage of samples and controls the general guidelines for assays detecting activation products were followed [11]. Ethylenediaminetetraacetic acid (EDTA) plasma samples from 40 healthy blood donors were separated immediately, cooled on crushed ice, pooled and stored in small aliquots at −70 °C. This normal human plasma pool control was called NHP. Individual blood donor samples (n = 20; 10 male and 10 female) and patient samples were obtained accordingly. Low control was a dilution of NHP, and high control was a dilution of the standard.

*General performance of EIA.* In a sandwich EIA, the wells of certified Maxisorp NUNC-Immunoplates (NUNC A/S, Roskilde, Denmark) were coated with 50 μl of purified MoAb Kok 12 at a concentration of 2μg/ml in 0.1 M carbonate buffer, pH 9.6, by overnight incubation at 4 °C. All other incubation steps were performed at 37 °C. After each incubation the wells were washed in 200 μl PBS with 0.1% Tween 20 (Sigma, St. Louis, MO, USA) three times. The samples and standards were diluted in PBS containing 0.2% Tween 20 and 10 mM EDTA, tested in triplicate, and incubated for 1 h. After washing, the plates were incubated for a further 45 min with a cocktail of goat anti-human C1r and goat anti-human C1s diluted in PBS containing 0.2% Tween 20 and 1% dried milk (Molico, Nestle, Vevey, Switzerland). Finally peroxidase-linked anti-goat IgG was diluted in PBS containing 0.2% Tween 20 and 1% dried milk. Substrate was 0.3 mM 2,2-azino-di-(3-ethyl)-benzthiazoline sulfonic acid (ABTS, Boehringer Mannheim, Mannheim, Germany) in 0.15 M acetate buffer, pH 4.0 and H₂O₂ to a final concentration of 2.4 × 10⁻³ M. Optical density was determined after 20–30 min on a Dynatech MR7000 reader at 410 nm.

*Quantification of C1-inhibitor.* C1-inhibitor antigen was quantified using the Nor-Partigen assay (Behringwerke, Marburg, Germany) performed according to the manufacturer’s instruction.

**RESULTS**

*Design of the assay.*

The MoAb Kok 12 is specific for a neoepitope exposed in C1-inhibitor when complexed with one of its proteases [7]. A double-antibody EIA with the MoAb Kok 12 as the capture and a cocktail of the polyclonal anti-C1r and anti-C1s as the detection antibody was constructed as described in materials and methods. Optimal dilutions of the reagents giving the highest signal to noise ratio were as follows: MoAb Kok 12 (1 mg/ml) diluted 1:500, serum activated with HAIGG used as standard diluted from 1:400 (two-fold, six step). Normal plasma pool and patient samples were diluted 1:100, low control was NHP diluted 1:200, high control was serum activated with HAIGG diluted 1:800. A cocktail of anti-C1r and anti-C1s was made by diluting the antibodies 1:1000 and 1:2000, respectively. Anti-goat IgG-peroxidase was diluted 1:2000.

The specificity of the double-antibody assay was examined by replacing the coating antibody Kok 12 with a control MoAb of the same subclass, and performing the assay as described above. These experiments were made on the same plate by coating half of the plate with Kok 12 and the other half with an anti-C5 MoAb. Strong reactions showing dose–response were observed using dilutions of the standard on the
part of the plate where Kok 12 was used. In contrast, no reaction was seen where the anti-C5 MoAb was used as capture control (Fig. 1).

**Titration curves for standard, NHS and NHP**

Standard, NHS and NHP were titrated and the titration curves compared (Fig. 2). The experiments were made on the same microtiter well plate. Standard was diluted 2-fold, and NHS and NHP were diluted 4-fold. Dilutions of samples were plotted against optical density, and showed virtually parallel dose–response curves. The markedly higher amounts of C1rs-C1inh complexes in the standard compared with NHS and NHP are illustrated in Fig. 2.

**Examination of blocking activity in NHP**

Because a potential problem with conventional complex assays has been blocking activity owing to presence of individual non-complexed components in plasma, we diluted the standard in buffer as well as in NHP diluted 1:100. No blocking activity was observed in NHP (Fig. 3). The curves were closely parallel in the upper part of the curve. The higher values in NHP at the lower part of the curve correspond to the content of C1rs-C1inh complexes in NHP diluted 1:100.

**Content of C1rs-C1inh complexes in NHP and NHS**

The standard was defined to contain 1000 arbitrary units (AU)/ml. Experiments comparing NHP and NHS with the standard showed that NHP contained 32 AU/ml \((n = 6)\), corresponding to 3.2% of fully activated serum. NHS contained 57 AU/ml \((n = 6)\), corresponding to 5.7% of fully activated serum.

**Lower detection limit**

The lower detection limit was found by dilution of the standard towards background, approximating background at a value of 0.05 AU/ml (Fig. 1). This corresponds to a detection limit of 5 AU/ml in NHP as these samples are optimally diluted 1:100.

**Variation coefficients and reference range**

Intra-assay variation coefficients were 12% for low control (median value 11 AU/ml) and 13% for high control (median value 91 AU/ml), \(n = 12\) for both. Inter-assay variation coefficients were 12% for low control \((n = 6)\), 19% for high control \((n = 6)\) and 15% for NHP \((n = 8)\). The median values were 11, 57 and 23 AU/ml, respectively. In a population of 20 healthy blood donors, 10 female and 10 male, plasma levels showed no significant gender difference. The median value for the whole group was 24 AU/ml. The reference interval, defined by the 2.5 and 97.5 percentiles, was 16–33 AU/ml.
**Patient samples**

The plasma levels of six HAE type 1 patients were compared with 20 normals (healthy blood donors). There was no significant difference with respect to absolute levels of the C1rs-C1inh complexes. The concentration of C1-inhibitor in these patients was 0.07 (0.05–0.09) g/l (median and range), compared with 0.29 (0.22–0.33) g/l in the control group. Thus, the patients had only ≈25% of the C1-inhibitor level of normals.

Two patients with SLE had elevated plasma concentrations of C1rs-C1inh complexes at, respectively, 62 and 56 AU/ml.

**DISCUSSION**

Detection of the neoepitopes expressed only on activated components is, theoretically, the preferred method to assess complement activation. These epitopes reflect the degree of activation of the corresponding component and can be quantified directly without the influence of native components [4]. With other EIA methods developed for measuring C1-inhibitor complexes, the interference from C1q(r-s)₂ and (C1r-C1s)₂ is a major potential problem. In the EIA described by Mathews et al. [10], excess anti-C1s was utilized to avoid interference of the native C1s compounds. Our assay has excluded this problem by using the neoepitope-based antibody, specific for complexed C1-inhibitor. Dilution of the standard in NHP clearly showed that no blocking activity was present in plasma in this assay.

The validity of the assay relies on the specificity of the MoAb Kok12 as described by Nuijens et al. [7]. However, we had to exclude the possibility of unspecific binding in the present EIA. The control coating-experiments comparing the neoepitope-specific antibody with a control antibody from the same subclass showed that unspecific binding was negligible.

The present assay has a sufficient sensitivity. The lower detection limit was 0.005% of activated serum, corresponding to 5 AU/ml in plasma diluted 1:100. The amount of complexes present in a normal human plasma pool was 32 AU/ml, equivalent to 3.2% of activated serum. Thus, normal human plasma diluted 1:100 showed six times higher values than the corresponding lower detection limit. The sensitivity of this assay cannot be compared with previous assays as these were not shown. Our assay has excluded the use of radioactive chemicals, and it is much more efficient (analysis time of 3–4 h) compared with previous published EIAs (Mathews et al. [10]: >2 days; Nuijens et al. [7]: >1 day). A further advantage of the EIA is the higher sample capacity and a low antiserum consumption. The inter- and intra- variation coefficients were also satisfactory.

The samples from the HAE patients were all obtained during remission. Theoretically, these heterozygous patients should have ≈50% of normal C1-inhibitor concentration. The fact that they have the same amount of C1rs-C1inh complexes, because of normal C1r and C1s, implies that HAE patients have to spend the same amount of C1-inhibitor as normals to keep C1 activation under control. This amount is taken from the already low C1-inhibitor levels in HAE patients and may partly explain why HAE patients in general have only 25–30% of normal C1-inhibitor concentration.

Only one previous study has reported the amount of C1rs-C1inh complexes in units comparable with ours [10]. They found 37.8 units/ml in normal plasma and 53.6 in serum, corresponding very closely to 32 and 57 found in our study. The standard was not the same in these two studies, but were made according to the same procedure.

Increased concentrations of C1-inhibitor complexed with C1r and C1s were found in the plasma of two patients with SLE. This is in agreement with earlier studies [12] and accords with the notion that one of the characteristics of SLE is classical complement pathway activation.

Mannose-binding lectin (MBL) provides an antibody- and C1-independent pathway for the activation of C4 and C2; the lectin pathway [13]. C1-activation and usually lectin pathway activation result in formation of the C3 convertase C4b2a. Analysing C4-activation has been used as a specific index of classical complement activation and reflects both C1-activation and in most cases activation through the lectin pathway. The present C1rs-C1inh assay is specific for C1-dependent activation and thus may discriminate between activation of the classical and the lectin pathway.

In conclusion, we have described a neoepitope-specific, fast, reliable, sensitive and non-radio labelled assay for selective detection of early classical pathway activation. It is suggested that this assay should be preferred for this purpose, and that it may prove to be useful for clinical studies.

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