Hemolytic assay for the measurement of functional human mannose-binding lectin: A modification to avoid interference from classical pathway activation

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1. Introduction

Low levels of functional mannose-binding lectin (MBL), impairing complement activation via the lectin pathway, result from single nucleotide polymorphisms in the MBL2 gene and have been associated with a variety of infectious and autoimmune diseases (Turner, 1996; Turner and Hamvas, 2000). Therefore, diagnostic procedures to determine MBL levels and lectin pathway activity are widely performed as part of immune status assessment.

In a previously described hemolytic assay, the lectin pathway of complement is activated by binding of MBL to mannan on Saccharomyces cerevisiae (Kuipers et al., 2002). Bystander-hemolysis of indicator erythrocytes is measured and compared to a standard sample to calculate the level of MBL. In this assay, all down-stream complement components are provided by addition of MBL-deficient serum. Therefore the rate limiting step is the amount of functional MBL in the test sample, independent of deficiencies or depletion of down-stream complement components. As both the ligand-binding and complement-activating properties of MBL are assessed simultaneously, this assay can quantitatively measure the level of functional MBL.

Interference by classical pathway activity in functional MBL assays has been postulated (Roos et al., 2003). In the hemolytic MBL assay described above, immune complexes could lead to MBL-independent complement activation, for example by antibodies against S. cerevisiae. Such interference could lead to false positive test results in patient samples. Consequently MBL deficiency will not be recognized and these patients will be misdiagnosed.

Abbreviations: MBL, mannose-binding lectin; VBS, veronal-buffered saline; HPS, human pooled serum.
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series of patients with an MBL-deficient genotype (XA/0 or 0/0) but with hemolytic activity in the hemolytic MBL assay. We have used those serum samples to modify the hemolytic assay. Here we describe the effect of classical pathway interference in the hemolytic MBL assay and the modification of this assay to prevent this artifact by addition of anti-C1q antibodies blocking classical pathway activity.

2. Materials and methods

2.1. Human blood samples

Serum and EDTA blood samples from 11 patients with an MBL-deficient genotype (XA/0 or 0/0) but activity in the unmodified hemolytic MBL assay (see below) were used. Furthermore, samples from 18 patients with MBL-sufficient genotypes (A/A and YA/0) were used. All samples were originally sent to the laboratory for genotyping and functional characterization of MBL as part of immune status assessment. MBL-deficient serum was obtained from a volunteer with MBL-deficient genotype 0/0. Human pooled serum (HPS) was prepared from serum of 20 healthy unrelated volunteers. All sera were stored at −70 °C.

2.2. Genotyping of MBL2

Combined haplotypes of single nucleotide polymorphisms (SNP) in the promoter and exon 1 of MBL2 were determined in a previously described denaturing gradient gel electrophoresis (DGGE) assay (Wiertsema et al., 2006). MBL2 genotypes were considered MBL-deficient (XA/0 and 0/0) or MBL-sufficient (A/A and YA/0) (Garred et al., 1999; Madsen et al., 1995).

2.3. Blocking of classical pathway activity by anti-C1q in the CH50 assay

To determine the concentration of anti-C1q needed to inhibit classical pathway activity, classical pathway complement activity was assessed in the presence of anti-C1q monoclonal antibodies in 4 randomly chosen sera. The sera were diluted 1/10 in veronal-buffered saline with 0.15 mM Ca2+ and 0.5 mM Mg2+ (VBS++ buffer) at a final volume of 100 μl containing 0, 5.5, 17, 50 or 150 μg/ml anti-C1q monoclonal antibodies mAB 2204 (Sanquin, Amsterdam, The Netherlands) (Roos et al., 2001) and preincubated on ice for 15 min. Complement mediated hemolysis of chicken erythrocytes was measured by spectrophotometry (absorbance at 405 nm). Hemolysis of the test samples was expressed as a percentage of hemolysis compared to HPS. Hemolysis <25% was considered deficient (Kuipers et al., 2002).

2.5. Determination of MBL protein levels

Serum levels of the multimeric MBL protein were determined in a commercially available ELISA (Sanquin, Amsterdam, the Netherlands) (Frakking et al., 2006). In short, MBL bound to coated mannann was quantified with the use of an anti-MBL antibody recognizing the multimeric form only.

3. Results

Anti-C1q antibody mAb 2204 inhibited classical pathway complement activity dose-dependently in four random sera, with full inhibition at 50 μg/ml (Fig. 1). Therefore, the 50 μg/ml anti-C1q antibody concentration was chosen to inhibit all classical pathway activity in subsequent experiments with the hemolytic MBL assay. All 18 samples from individuals with MBL-sufficient genotypes A/A or YA/0 showed hemolysis >25% in the unmodified hemolytic MBL assay, i.e. without anti-C1q addition (Fig. 2). Only 1 of these samples (genotype YA/0) showed deficient hemolysis (<25%) upon addition of anti-C1q. The MBL protein level in this sample was <0.2 μg/ml.

A striking result was found in sera from 11 patients with MBL-deficient genotypes XA/0 and 0/0. In the unmodified assay in absence of anti-C1q, hemolysis exceeded 25% in all of these samples, suggesting functional MBL activity (Fig. 2).

Fig. 1. Dose-dependent inhibition of classical pathway mediated hemolysis in the CH50 assay by anti-C1q antibody mAb 2204. The mean hemolysis (OD 405 nm) of four randomly selected sera is depicted. Classical pathway mediated hemolysis was completely inhibited at 50 μg/ml.
Addition of anti-C1q completely inhibited hemolysis in all samples, indicating the absence of functional MBL activity in these sera. MBL protein levels were below 0.2 μg/ml in all samples with MBL-deficient genotypes.

Measurements in the modified hemolytic assay were linearly correlated with the MBL protein levels (Spearman’s rho 0.969, p < 0.001).

**4. Discussion**

The hemolytic MBL assay is designed to measure functional MBL levels by the ability of MBL in the test serum to bind its ligand and to activate complement, independent of depletion or deficiencies of downstream complement components (Kuipers et al., 2002). It is based on the principle of bystander hemolysis. Because binding of C1q to immune complexes and subsequent classical pathway activation would also result in hemolysis, classical pathway activity could interfere with the hemolytic MBL assay.

The anti-C1q monoclonal antibody mAb 2204 inhibited classical pathway activity dose-dependently in the CH50 assay and thus should be able to prevent classical pathway interference in the MBL assay. The concentrations of anti-C1q found to completely inhibit classical pathway activity in our study are in the same range as the suggested 20 μg/ml by Roos et al. (2003). Since the anti-C1q antibody is directed against the globular head of C1q and inhibits the binding of C1q to IgG, its blocking effect is specific for the classical pathway activity (Hoekzema et al., 1988; Roos et al., 2001).

In clinical samples, the classical pathway interference could lead to an overestimation of MBL-mediated hemolysis. Especially in patients with MBL-deficient genotypes, this interference would lead to a false test result of sufficient functional MBL levels. Addition of 50 μg/ml anti-C1q completely inhibited this artifact, as the functional results of the modified MBL assay were in complete accordance with the MBL protein levels of these individuals and no hemolytic activity was found in patients with MBL-deficient genotypes.

In conclusion, the hemolytic MBL assay can measure functional MBL levels without interference from downstream complement deficiencies. Classical pathway interference in this and other functional MBL assays can and should be blocked by anti-C1q to prevent that MBL deficiency is overlooked and patients are misdiagnosed.

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