Clinical application of new technique that measures C4d for assessment of activation of classical complement pathway

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SUMMARY A new laser nephelometric technique that measures C4d for the assessment of the activation of the classical complement pathway was developed. C4d was isolated from other larger C4 related molecules at a final concentration of polyethylene glycol of 12% and then quantitated by laser nephelometry using a commercially available antiserum, which reacts with C4d determinants. C4d standard (100%) was produced by exhaustive activation of the classical pathway in pooled normal human serum using heat aggregated human immunoglobulin. Serial dilutions of the standard provided a reference curve against which clinical samples were read. Patients with rheumatoid arthritis showed significantly higher C4d values (mean 53.8%) than controls (21.7%; $p < 0.001$). The technique proved accurate, rapid, and suitable for the routine laboratory evaluation of complement activation through the classical pathway, and it may be useful in the management of those conditions in which complement activation has a pathogenic role.

A common feature of connective tissue disorders is tissue damage caused by activation of the complement system.¹ Detection and quantitation of complement activation may be useful for monitoring disease activity.²

The laboratory assessment of complement activation is usually based on the circulating concentrations of C3 and C4.³ Values below the normal range are assumed to be due to consumption; but such an assumption can be misleading when used as an assessment of complement activation. Variations in the concentrations of complement factors can occur for reasons other than consumption. In rheumatoid arthritis and in the seronegative arthritides both C3 and C4 serum concentrations are often increased due to their behaviour as acute phase reactants.⁴ Conversely, the low concentrations of C4 in systemic lupus erythematosus may be genetically determined due to the possession of null C4 allotypes.⁵

Complement activation can be incontrovertibly shown by measuring complement fragments, which are generated during complement activation.³ A nephelometric technique for the determination of the fragment C3d in plasma, following its isolation in polyethylene glycol (PEG), has recently been des-

cribed.⁶ Raised C3d concentrations, however, do not indicate the activation route, as both classical and alternative complement pathways converge at C3 level with production of C3d (fig 1).⁷ C4d is a low molecular weight (44 500) fragment cleaved from C4 during activation of the classical complement pathway (figs 1 and 2).⁸ Its measurement may therefore provide an estimation of the classical pathway activation.

We report a nephelometric technique for the quan-

Fig 1 Diagram of complement pathways. Activation of both classical and the alternative pathway leads to splitting of C3 with production of C3d. IC = Immune complexes. LPS = lipopolysaccharides.

Fig 2 Schematic representation of cleavage of C4.

titration of C4d in plasma. The clinical relevance of the assay was investigated by determining C4d concentrations in healthy subjects and in patients with rheumatoid arthritis, where activation of the classical complement pathway is known to occur.^{9,10}

Material and methods

Twenty healthy subjects (nine men, median age 31, range 22–53) and 50 patients (10 men, median age 48, range 16–72) with classical or definite rheumatoid arthritis, defined according to the criteria of the American Rheumatism Association, were studied. Blood collected into a final concentration of 10 mmol/l edetic acid was immediately centrifuged at 1000 g at 4°C for 15 minutes; the plasma was separated and stored at –70°C.

C4d STANDARD

A pool of normal human sera was incubated at 37°C for 60 minutes with 5 mg/ml of heat aggregated human immunoglobulin (HAGG), which exhaustively activates the classical pathway.¹¹ HAGG was prepared by heating a purified Cohn fraction II (Sigma Chemicals, Poole, Dorset) at 63°C for 20 minutes.¹² After centrifugation of the activated serum at 1500 g for 30 minutes the C4d rich supernatant was recovered, aliquoted, and stored at –70°C. This preparation was used throughout the study as the source of C4d standard.

PEG ISOLATION OF C4d

To isolate C4d from native C4 and the larger C4 fragments 100 µl of C4d standard were added to 100 µl of PEG 6000 (Sigma Chemicals) in borate buffer saline (BBS), pH 8.3, 0.1 mol/l, to achieve final concentrations ranging between 6–18%. After incubation at 4°C for 90 minutes these preparations were spun down at 1500 g at 4°C for 30 minutes and the supernatants recovered. The efficacy of various PEG concentrations in separating C4d was assessed by immunoelectrophoresis performed in 1.2% agarose 15 (BDH Biochemicals, Poole, Dorset) in barbitone buffer, pH 8.6, 45 mmol/l, at 10 V/cm for 60 minutes. Optimal PEG concentration was considered to be that removing any slow migrating C4 moiety, but leaving in solution a fragment migrating in the alpha region (C4d).^{12,13}

SELECTION OF ANTISERUM

As no specific anti-C4d antiserum is commercially available, several anti-C4 antisera were tested for their ability to detect C4d. These included anti-C4 antisera from: Atlantic Antibodies (American Hospital Supply, Compton, Berkshire); Dakopatts (Dakopatts, High Wycombe, Buckinghamshire); Kallestad (Kallestad Laboratories, Brill, Buckinghamshire); Immuno (Immuno Diagnostics, Sevenoaks, Kent); Northeast (Northeast Biomedical Laboratories, Uxbridge, Middlesex); Pel-Freez (Pel-Freez Clinical Systems, Uxbridge, Middlesex); and Seward (Seward Laboratories, London). Two further antisera by Behring (Behring Diagnostics, Hounslow, Middlesex) were also tested—anti-C4 laser nephelometry grade antiserum and anti-C4 intact antiserum. An antiserum was considered to be reactive with C4d if capable of forming a precipitin arc in the alpha region, when tested by immunoelectrophoresis against a preparation containing fragments derived from the cleavage of C4 (C4d standard).

MEASUREMENT OF C4d BY LASER NEPHELOMETRY

To define the optimal conditions of the assay a checker board titration was performed. supernatant

Fig 3 Immunelectrophoresis of C4 and its split products. Well a: normal edetic acid plasma; well b C4d standard (pooled normal human serum activated by heat aggregated human immunoglobulin); well c supernatant recovered after PEG treatment of C4d standard at a final concentration of 12%; troughs anti-C4 intact antiserum by Behring. Anti-C4 antiserum reacts with alpha migrating fragment contained in C4d standard (b) which is still present (arrow) after PEG treatment (c), while slower C4 related molecules are successfully removed by treatment with PEG at a final concentration of 12% (c). No alpha migrating fragment can be detected in normal edetic acid plasma (a) where only intact C4 is identified.

from PEG treated C4d standard was serially diluted in BBS and incubated at room temperature with different dilutions in BBS of the anti-C4 antiserum. Readings were taken in a Behring laser nephelometer at 30, 60, 90, 120, and 180 minutes and compared with a reference curve prepared from C4d standard.

Clinical data are presented as mean $\pm$ 2 SD. The difference between mean values was evaluated using Student's *t* test.

Results

PEG ISOLATION OF C4d AND ANTISERUM SELECTION

A final concentration of PEG of 12% separated C4d from larger C4 related molecules (fig 3). At this concentration of PEG larger C4 molecules were completely removed by centrifugation and the smaller C4d remained in the supernatant. Higher PEG concentrations also tended to precipitate C4d. The antiserum best able to detect C4d was anti-C4 intact antiserum (Behring Diagnostics), which gave a distinct precipitin line in the alpha region (fig 3). This antiserum was therefore used for the study.

MEASUREMENT OF C4d BY LASER NEPHELOMETRY

The optimal conditions for the measurement of C4d by laser nephelometry were as follows. After isolation of C4d by precipitation at 12% PEG supernatants from test samples were diluted 1/2.5 in BBS and added to an equal volume of anti-C4 antiserum diluted 1/5 in BBS. The final PEG concentration was thus reduced to

2.4%. The top point of the reference curve was obtained by diluting the supernatant of the C4d standard (containing 12% PEG) 1/2.5 in BBS. This first dilution leads to a PEG concentration of 4.8%. The remaining points of the reference curve were derived by double dilution of the top standard to 1/80 in 4.8% PEG in BBS (fig 4). The addition of 1/5 antiserum to all the supernatant dilutions brought the final PEG concentration to 2.4%. This detail is important as PEG influences the kinetics of immuno-precipitation reactions.¹⁴ C4d/anti-C4d reaction was read in the nephelometer after 90 minutes' incubation.

C4d could also be measured under different experimental conditions. Final concentrations of PEG between 2.0% and 3.0% were found to be suitable for the performance of the assay; higher and lower concentrations decreased its sensitivity. The final PEG concentration of 2.4% was adopted as it offered the best discrimination between small changes of C4d concentration. For similar sensitivity reasons the dilution of the anti-C4 antiserum chosen was 1/5. The nephelometric readings of C4d/anti-C4d reaction became stable at 90 minutes, remaining stable thereafter. Ninety minutes, therefore, was chosen as the optimal period of incubation.

The intra-assay coefficient of variation derived from determining the same sample 30 times was 6.0%. The interassay coefficient of variation derived by repeating the estimation of the same sample on 10 different occasions was 7.5%.

C4d concentrations in 20 healthy subjects and in 50 patients with rheumatoid arthritis are shown in fig 5.

Fig 4 Reference curve derived from serial dilutions of supernatant of C4d standard from 1/2.5 to 1/80. Top dilution is given a value of 100%.

C4d values are expressed as a percentage, the C4d standard being 100%. Patients with rheumatoid arthritis presented with C4d values (mean 53.8%, SD 23.7%) significantly higher than those of controls (21.7% SD 10.0%); $p < 0.001$). Twenty nine of the 50 (58%) patients had C4d concentrations above the upper limit of normal (mean control value + 2 SD).

Discussion

We established a new technique measuring C4d for the assessment of activation of the classical complement pathway. C4d values were found to be increased in most of the patients with rheumatoid arthritis, a typical immune complex disease,¹⁵ in which the classical pathway is activated.^{9,10}

Numerous assays aimed at assessing activation of the classical complement pathway have focused on the breakdown of C4, a component exclusive to this pathway. Turnover studies based on the administration of radiolabelled C4, have provided evidence of hypercatabolism of the molecule in immune complex diseases, such as seropositive and seronegative arth-

ritides.¹⁶ The invasive nature of the technique, however, precludes its routine clinical application.

Unequivocal evidence of classical pathway activation can be obtained through C4 breakdown products. C4 cleavage products have charge and molecular weight differences from intact C4 and they can therefore be identified by electrophoretic techniques, including immunoelectrophoresis,¹⁷ counter immunoelectrophoresis,¹⁸ and crossed immunoelectrophoresis.¹⁹ These techniques are insensitive and mainly provide qualitative results. Moreover, they are time consuming, laborious, and hardly suitable for analysis of large sample batches. Recently, rocket immunoelectrophoresis has been proposed for the quantitation of fragment C4d.^{20,21} This assay also seems to be insensitive, failing to detect the concentrations of C4d present in normal subjects; and it is cumbersome, requiring photographic enlargement for interpretation of results.

Fig 5 C4d in plasma of normal controls and patients with rheumatoid arthritis. Values are expressed as per cent of C4d standard. Horizontal bars represent mean values. Dotted line indicates upper limit of normal (mean control value + 2SD). Patients with rheumatoid arthritis have significantly higher C4d values than controls ($p < 0.001$).

Products of the partial proteolysis of C4 can be identified by exploiting their solubility in PEG. High PEG concentrations precipitate intact complement factors and their heavier breakdown products, leaving lighter fragments in the supernatant where they can be quantitated. A radial immunodiffusion technique to measure PEG isolated C4d fragment has been reported.¹² This technique lacks sensitivity, is lengthy, and has never been reproduced because of the unavailability of commercial antisera specific for C4d.

Lack of reactivity for C4d seems to be a common feature of commercial anti-C4 antisera, probably reflecting the weak immunogenicity of the C4d fragment. In this study only one of nine antisera tested could recognise C4d. Using this antiserum we developed a laser nephelometric technique to measure C4d after its isolation in high concentration of PEG. This assay is sensitive, permitting the detection of small amounts of C4d present in normal subjects; precise, having low intra- and interassay coefficients of variation; rapid, providing results within four hours; and simple to perform. Furthermore, the steps following PEG separation of C4d can be fully automated.

The present study also shows that the measurement of C4d is of clinical value. The increased C4d concentrations found in most patients with rheumatoid arthritis provide clear evidence for classical complement pathway activation in this immune complex disease. Such activation could have been missed if intact C4 had been determined, as the concentrations are usually normal or increased in patients with rheumatoid arthritis, due to the behaviour of C4 as an acute phase reactant.^{4,22}

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