Clinical application of a new nephelometric technique to measure complement activation

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SUMMARY A new nephelometric technique to measure C3d as an indicator of complement activation, is described. C3d is isolated at high concentration of polyethyleneglycol (PEG), incubated with commercially available anti-C3d antiserum at a final concentration of 2.5% PEG and then measured in a Behring Laser Nephelometer.

In contrast to previously available techniques our assay detects the low concentrations of C3d present in all normal subjects, which result from the continuous C3 catabolism occurring *in vivo*.

We have also measured C3d blood concentrations in a large number of patients with diseases associated with complement activation. Raised C3d concentrations were found in 68% of rheumatoid arthritis, 57% of primary biliary cirrhosis, 38% of chronic active hepatitis, 100% of Gram-negative bacteraemia and 100% of malaria.

The nephelometric technique has proved to be sensitive, economical and fast, and could be adapted for routine determination of C3d blood concentrations to monitor disease activity and response to treatment.

In a number of immunologically mediated disorders, the occurrence and degree of tissue damage depends on the activation of the complement (C') system.^{1–3} To detect and measure C' activation is useful not only for assessment of disease activity but also to monitor response to treatment.

The complement cascade can be activated via a "classical" pathway following an antigen-antibody reaction or via an "alternative" pathway which usually does not require an antibody as triggering factor.^{4–6} Both pathways converge at the level of C3 whose split products reflect C' activation by either pathway. C3 is broken down by C3 convertases to a small C3a and a large C3b fragment. C3b is inactivated by C3b inhibitor (factor I) to form C3bi. A further trypsin-like enzymatic activity produces C3c and C3d fragments (Fig. 1).

The electrophoretic mobility of C3 fragments differs from that of native C3, and this has been used to assess C' activation. Several techniques have been devised which exploit this different mobility of the C3 fragments, and include immunoelectrophoresis,⁷ crossed immunoelectrophoresis,⁸ counter immunoelectrophoresis,⁹ and immunofixation.¹⁰ All these methods are mostly qualitative, time-

consuming and hardly suitable for screening large batches of samples. Furthermore, some—that is, crossed immunoelectrophoresis and immunoelectrophoresis—require large amounts of antisera.

An alternative approach has been reported by Perrin *et al*¹¹ who have investigated the solubility of several C' components and fragments at increasing concentrations of polyethyleneglycol (PEG). These authors have shown that of the C3 split products only C3d was present in solution at concentrations above 11% of PEG: this fragment was measured by radial immunodiffusion, a technique that requires at least 48 h before reading.

We decided to measure C3d by laser nephelometry, a technique faster and more sensitive than radial immunodiffusion. C3d was chosen as measure of C' activation for two reasons: (i) the presence of C3d in the circulation is unequivocal proof of C' activation; (ii) C3d in contrast to other C3 breakdown products is metabolised slowly¹² and thus provides a longer lasting trace of C' activation. After attaining optimal assay conditions with normal human plasma, a normal range in a large control population has been established. We then tested the potential clinical use of the assay in a substantial number of patients suffering from conditions where complement is activated by either pathway.

Fig. 1 Diagram of C3 breakdown. C3 convertases split C3 into C3a and C3b fragments; the latter is inactivated by factor I and the resulting C3bi is further cleaved into C3c and C3d by enzymatic activities contained in normal human serum.

Material and methods

REAGENTS

Borate buffer, 0.5 M, pH 8.2.
 22% polyethyleneglycol MW 6000 (PEG 6000) in borate buffer.
 5% PEG 6000 in borate buffer.
 1.1% PEG 6000 in borate buffer.
 Anti-C3d antiserum (Dakopatts-Copenhagen).
 Anti-native C3 antiserum (Immuno).
 Agarose 25 (BDH Biochemicals).
 Barbitone buffer. (0.065 M pH 8.6).

PLASMA

Blood was drawn by venepuncture at a final concentration of 10 mmol/l EDTA. Plasma was immediately separated by centrifugation at 4°C and stored at -70°C. Serum was also separated from clotted blood and stored at -70°C.

Samples were obtained from 45 healthy control subjects, 108 patients with classical or definite rheumatoid arthritis, 28 with primary biliary cirrhosis, 52 with chronic active hepatitis, 20 with Gram-negative bacteraemia and four with malaria.

PRELIMINARY EXPERIMENT ON C3d

GENERATION AND ON C3d SOLUBILITY IN PEG
 Complement was exhaustively activated through the alternative pathway by adding 3 mg of inulin to each ml of a pool of freshly drawn normal human sera (NHS). To separate C3d thus produced from native C3, C3b and C3c, one millilitre of inulin-treated

NHS was mixed with 1 ml of ice cold 22% PEG 6000 in borate buffer, incubated for 60 min at 4°C and spun at 1500 g for 30 min at 4°C.¹¹ The supernatant was then recovered, and tested in immunoelectrophoresis (1% agarose in barbitone buffer) against anti-native C3 and anti-C3d antisera.

A single precipitin arc in α -region was obtained with anti-C3d but not with anti-native C3 antiserum. At a final concentration of 11% PEG only an α -migrating fragment is still in solution and is recognised by anti-C3d antiserum (Fig. 2). Although we have maintained the term C3d for the fragment detected, since it is recognised by commercially available anti-C3d antiserum, its electrophoretic mobility is similar to that of so-called C3d.g recently identified by Lachmann *et al.*¹³ These authors using a panel of monoclonal anti-C3 antibodies have shown that the final product of C3 activation in vivo, as detected by commercially available anti-C3d antiserum, not only contains C3d determinants but also a newly recognised determinant called C3g.

DEVELOPMENT OF A NEPHELOMETRIC ASSAY FOR MEASUREMENT OF C3d

Inulin-treated NHS was used as 100%-C3d reference standard and, suitably diluted, provided standard curves. C3d was separated as described above, at 11% final concentration of PEG 6000. In brief 100 μ l of EDTA-plasma (or serum) were mixed with 100 μ l of 22% PEG, incubated for 60 min at 4°C and spun at 1500 g for 30 min at 4°C. The C3d containing supernatant was then recovered.

To establish the working conditions of the assay a chess-board titration of antigen (C3d containing supernatant) against antibody (anti-C3d antiserum)

Fig. 2 Immunoelectrophoresis of the C3 split products soluble in 11% PEG.

Well a: normal EDTA plasma.

Well b: inulin activated normal human serum (NHS).

Well c: supernatant of the inulin activated NHS after PEG treatment.

Trough 1: anti-native C3 antiserum.

Trough 2: anti-C3d antiserum.

Anti-C3d antiserum reacts with an α -migrating fragment, contained in the inulin activated NHS (b) which is still present (arrow) after PEG treatment (c). Anti-native C3 fails to react with this fragment.

was performed at several final concentrations of PEG 6000. Readings were taken in a Behring Laser Nephelometer at 15, 30, 60, 90, 120, 150, 180 min and 24 h and referred to a standard curve prepared from 100%-C3d standard. Antigen/antibody complexes produce a scatter of light when hit by a laser beam: this generates a voltage readable on the Nephelometer display. When concentrations of antibody and of PEG are constant, the voltage varies proportionally to the concentration of antigen.

Results

C3d could be detected under different experimental conditions. Readings were obtained with up to 1/10; dilution of the antiserum, but 1/5 dilution was chosen because it produced a more sensitive standard curve (Fig. 3). Final concentrations of PEG between 0.75% and 2.5% proved to be suitable for detection of the antigen: higher and lower concentrations decreased the sensitivity of the assay while no reading was obtained when PEG was not added (Fig. 4). Comparable results were obtained when readings were performed at times varying between 15 and 180 min; we found it more convenient to read after 60 min incubation (Fig. 5). In summary optimal conditions were obtained mixing 100 μ l of C3d—containing supernatant, diluted 1/10 in borate buffer, with 100 μ l of anti-C3d antiserum diluted 1/5 in 5% PEG. The final PEG concentration was approximately 2.5%. Readings were taken at 60 min. The standard curve was obtained by first diluting the

Fig. 3 Effect of anti-C3d dilution on the detection of C3d at a final PEG concentration of 2.5%. 1/5 dilution gives a more sensitive standard curve.

Fig. 4 Effect of different concentrations of PEG on C3d detection using anti-C3d antiserum diluted 1/5.

Fig. 5 Effect of time on the nephelometric reading of C3d-anti-C3d reaction at an anti-C3d dilution of 1/5 and PEG final dilution of 2.5%.

100%-C3d standard 1/10 in borate buffer and then double diluting five times in 1.1% PEG.

Low yet measurable concentrations of C3d were present in all the 45 normal subjects tested. C3d values were expressed in arbitrary nephelometric units. The mean of the values obtained on nor-

Fig. 6 C3d concentrations in healthy individuals and in five disease states associated with complement activation.

mal+2 SD provided the upper limit of normal for the assay (Fig. 6).

C3d concentrations in patients suffering from conditions where complement is expected to be activated via either pathway is shown in Fig. 6. Total number and percentage of patients with raised concentrations of C3d are as follows: rheumatoid arthritis 73/108 (68%), primary biliary cirrhosis 16/28 (57%), chronic active hepatitis 20/52 (38%), Gram-negative bacteraemia 20/20 (100%) and malaria 4/4 (100%).

Discussion

We have established a new nephelometric technique to measure complement activation at the level of C3, the component of the complement system central to both pathways of activation. The fragment measured is soluble at high concentrations of polyethyleneglycol and migrates electrophoretically in α -region.

Using our nephelometric technique we have shown that measurable concentrations of C3d are present in all the healthy subjects studied. This is in contrast with previous reports where C3d was

detected in only about 50% of normal controls,¹¹ probably a reflection of a less sensitive technique. The findings of measurable concentrations of C3d in healthy individuals is not surprising since, regardless of the presence of activating substances, a "priming" C3 convertase is slowly assembled, continuously providing small amounts of breakdown products.⁶ This ability to measure C3d concentrations on all normal subjects allows us to establish a normal range (mean $\pm$ 2 SD) and to obtain a more meaningful statistical analysis.

In addition, nephelometry is a relatively simple technique, which permits the testing of a large number of samples in a relatively short time (about two hours). The test described is also economical since 20 μ l of anti-C3d have been efficiently used for C3d measurement and as little as 10 μ l can still provide recordable results.

The application of the nephelometric technique to measure C3d in a large series of patients affected by diseases which have been associated with complement activation has provided interesting observations. We have found raised concentrations of C3d in 68% of the patients with rheumatoid arthritis. In 99 of these patients, in whom C1q binding and C3 concentrations were also tested, there was a highly significant correlation between level of immune complexes, as measured by C1q binding, and complement activation as detected by C3d.¹⁴ In spite of complement activation, C3 blood concentrations were normal or increased, probably reflecting the known behaviour of early complement components as acute phase reactants.¹⁵ In a similar way total haemolytic complement activity has been found normal or slightly raised in the serum of patients with rheumatoid arthritis while it was significantly depressed in the joint fluid.¹⁶ Thus, intra-articular complement activation is probably the cause of increased plasma concentrations of C3d, which can freely diffuse from the joint due to its low molecular weight (35 000 daltons).

In liver disease, the interpretation of serum complement values is complicated by the fact that the liver produces most of the complement components. Indeed, in the presence of massive liver cell necrosis as in fulminant hepatic failure, dramatically reduced concentrations of complement fractions are associated with undetectable C3d concentrations (D Vergani, unpublished data 1982) indicating lack of synthesis. Controversial results have been obtained by different authors in chronic active hepatitis and primary biliary cirrhosis.¹⁷⁻¹⁹ When low concentrations of complement fractions were found, this was interpreted as the result of complement consuming immune reactions. Indeed, we have found evidence of complement activation in a high proportion of our

patients with primary biliary cirrhosis or chronic active hepatitis.

In Gram-negative bacteraemia low concentrations of C3, reflecting complement consumption, have been reported only when shock occurs.²⁰ However, measuring C3d we have shown complement activation in 100% of the cases, which reflects the high sensitivity of the nephelometric assay in detecting sustained activation of classical and alternative complement pathways by bacterial endotoxins.²¹

Finally our findings of high concentrations of C3d in all four cases of malaria studied is not surprising. Indeed in this condition complement can be activated by several mechanisms, which include soluble immune complexes, erythrocyte-antibody complexes and raised concentrations of endotoxins.²²

Our results show that the sensitivity of the nephelometric technique allows the assessment of complement consumption even in those conditions where haemolytic and immunochemical complement levels are normal or increased. Since the products of complement activation are major mediators of inflammatory tissue damaging processes, the possibility of detecting such activation might prove useful in the investigation of the pathogenesis of several disease states.

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