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Abstract

A sensitive assay for the functional activity of complement Factor I is described. This is based on its third proteolytic clip whereby Factor I cleaves cell-bound iC3b to cell-bound C3dg and soluble C3c, thereby abolishing conglutination of the cells. Factor H is required as a co-factor for Factor I activity. Because of the low affinity of iC3b for Factor H, the assay needs to be performed at low ionic strength. This assay is easier to perform than those based on the conversion of C3b to iC3b (the first two Factor I clips), there being no need for the unstable intermediate EAC142 or for purified C3.

Keywords	Factor I assay; complement C3;conglutination.
Taxonomy	Medical Tests, Immunopathology
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Suggested reviewers	Zvi Fishelson, veronique fremeaux-bacchi, Paul Morgan, Michael Kirschfink, John Atkinson, David Kavanagh

Submission Files Included in this PDF

File Name [File Type]

Cover letter 6 Mar 2018.docx [Cover Letter]

Response to Reviewers 6 Mar 2018.docx [Response to Reviewers]

Highlights 26 Mar 2018.docx [Highlights]

Factor I assay J Imm Meth 6 Mar 2018.docx [Manuscript File]

Figure 1 6 Mar 2018.pptx [Figure]

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Table 1 J Imm Meths 6 Mar 2018.xlsx [Table]

Table 2 I Imm Meths 6 May 2018.xlsx [Table]

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6 Mar 2018

Dear Michel,

Thank you for accepting the paper and thank the referees for their detailed appraisal. We have revised the manuscript to take account of all – or virtually all – their points. We have included quite a bit more background, including a new Figure, although I don't imagine that many people who are not fairly familiar with complement will be interested in a Factor I assay! I look forward to seeing you at the Kunkel Society meeting in Paris next month.

All best wishes.

Yours sincerely,

Peter

Response to Reviewers

We have responded to the reviewers suggestions by expanding the background and dealing with the individual points raised.

Highlights

A novel conglutinating assay for the functional activity of complement Factor I

Measuring the degradation of cell-bound iC3b to cell-bound C3dg and soluble C3c

Easier to perform than assays based on the conversion of C3b to iC3b

A novel and sensitive functional assay for complement Factor I based on the third proteolytic clip of C3b

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Abstract

A sensitive assay for the functional activity of complement Factor I is described. This is based on its third proteolytic clip whereby Factor I cleaves cell-bound iC3b to cell-bound C3dg and soluble C3c, thereby abolishing conglutination of the cells. Factor H is required as a co-factor for Factor I activity. Because of the low affinity of iC3b for Factor H, the assay needs to be performed at low ionic strength. This assay is easier to perform than those based on the conversion of C3b to iC3b (the first two Factor I clips), there being no need for the unstable intermediate EAC142 or for purified C3.

Keywords: Factor I assay; complement C3; conglutination

1. Introduction

Complement Factor I is the induced-fit enzyme responsible for the C3b breakdown cycle of the alternative complement pathway. Together with co-factors – Factor H, membrane co-factor protein (CD46) or CR1(CD35) – it breaks down C3b firstly to iC3b, a process which involves two proteolytic cleavages and then to C3c and C3dg with a final third clip (Figure 1). Elevated concentrations of Factor I powerfully down-regulate the complement alternative pathway (Lachmann and Halbwachs 1975) so that measurement of Factor I had become of clinical interest.

Sensitive functional assays for Factor I have in the past been done using an assay for the conversion of C3b to iC3b. iC3b reacts with the bovine plasma lectin – conglutinin (Lachmann 1962) - whereas C3b does not. This assay therefore measures Factor I activity for its first two proteolytic clips of C3b which give rise to the formation of iC3b (Figure 1). There are certain disadvantages to this assay. It requires, on each occasion, a supply of C3 to be reacted with EAC142[1] which is always a slightly exacting procedure since EAC142 is unstable and purified, native C3 has to be used. If the C3 is highly purified, some Factor H will need to be added.

[1] Erythrocyte/antibody reacted with complement components 1, 4 and 2

To avoid these difficulties, we have developed an assay that looks at the destruction of iC3b rather than its formation. EAC1423bi is easy to make and requires only EA[2] and a human "R3" reagent (made by absorbing fresh normal human serum with zymosan or a similar yeast cell preparation). The EAC1423bi is stable for long periods and can be shown to be suitable for the assay by demonstrating that it is readily agglutinated by conglutinin. The agglutination produced by conglutinin is extremely powerful and for this reason has always been described as "conglutination" rather than simply "agglutination". The destruction of iC3b by Factor I under physiological conditions requires the use of CR1 or a derivative thereof as a co-factor. These are not always readily available. However, it is well known that the failure of other Factor I co-factors, such as Factor H, to catalyze this last clip is due solely to their low affinity for iC3b. This can be overcome by reducing the ionic strength and in these circumstances Factor H is a perfectly satisfactory co-factor for the last split.

2. Materials & Methods

2.1 Reagents

Sheep red blood cells (E) in Alsevers (Oxoid, Product No. SR0053B, Oxoid Limited, Wade Road, Basingstoke, Hampshire, RG24 8PW, UK)

IgM anti-sheep E monoclonal antibody (S016 – in house). Commercial alternatives are available (for example from Complement Technology, Inc.**,** 4801 Troup Hwy, Suite 701, Tyler, Texas 75703, USA .

Zymosan (Sigma, Product No. Z4250)

Factor H and Factor I – Complement Technology, Inc.**,** 4801 Troup Hwy, Suite 701, Tyler, Texas 75703, USA. The concentration of Factor I was measured by A_{280} extinction coefficient taking 1.40 as extinction at 1 mg/ml Factor I (which includes the carbohydrate) (Harrison 1996).

Human R3 (see below)

Bovine conglutinin (prepared in house – see below)

Complement fixation buffer (CFT) (Oxoid)

Low ionic strength complement buffer is made by diluting one part CFT with 4 parts of 5% glucose.

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[2] Erythrocyte/antibody

EA is prepared in the standard way by treating sheep E with 5 minimal haemolytic doses of antibody SO16 (Harrison 1996). Pre-prepared EA is available commercially, for instance from CompTech Inc.

Human R3 is a reagent developed by the Pillemer laboratory in the 1950s (Pillemer et al 1954) depleted of "C'3", now known to be made up of C3 to C9, and is made by treating normal human serum (NHS) with zymosan (yeast cell walls) at 37^{o}C for 45 minutes as described in detail in Harrison (1996). Human R3 still contains some C3 but is depleted of Factor B and usually of C5 and C6.

EAC(1)4(2)3bi is prepared by treating EA with the R3 reagent for 30 minutes at 37^{o}C. This changes to EAC43bi upon release of C1 and C2a. The cells are then washed x 3 and finally resuspended to 1% in the low ionic strength complement buffer.

Factor H is added to a final concentration of 2µg/ml.

Conglutinin is prepared from 56^{o}C heated bovine serum by absorption onto zymosan and elution with EDTA (Lachmann 1962; Lachmann and Richards 1964; Harrison and Lachmann 1986). Alternatively, recombinant conglutinin may be obtained from MyBioSource, P.O. Box 153308, San Diego, California, USA.

Antigenic concentration of Factor I was measured using the radial immunodiffusion assay system from The Binding Site Group Ltd., 8 Calthorpe Road, Edgbaston, Birmingham, B15 1QT.

2.2 Titration of conglutinin

Using CFT with 2 mM calcium, the conglutinin was titrated on EAC43bi cells in a microtitre plate (50 µl 1% cells plus 50 µl conglutinin dilution). The plate was incubated at 4^{o}C until settled. The minimum conglutinating dilution was 1/800 and it was decided to use three conglutinating doses for the Factor I assay, i.e. 1/200. There are approximately 100,000 conglutinating units per gram conglutinin protein.

2.3 Factor I assay

Factor I was titrated using doubling dilutions of Factor I, starting at 8.45 µg/ml in low ionic strength buffer containing gelatin at 1 mg/ml in small glass tubes. 50µl 1% EAC43bi/ Factor H cells were added to each tube and incubated for 1 hour at 37^{o}C with gentle shaking. 50 µl conglutinin (at 1/200 conglutinin) was then added to each

tube and the mixture incubated at 4^{o}C for at least one hour or until the cells had settled. Controls were EAC43bi and no Factor H; Factor I diluted in CFT/gelatin plus Factor H (but not low ionic strength).

The conglutination can be read by settling pattern or, if preferred, by centrifuging the cells and resuspending them. If the cells remain conglutinated it will appear as clear liquid with clumps of cells in suspension. Loss of conglutination results in a cloudy liquid. In this assay it is loss of conglutination that is looked for as evidence of Factor I activity.

The concentration of Factor I in normal human sera was measured using doubling dilutions in low ionic strength buffer using the above assay protocol. Sera collected from three different individuals was heat inactivated at 56^{o}C for 30 minutes before use. The control was serum with no added Factor H.

3. Results and Dicussion

The results of the titrations of Factor I and controls are summarised in Table 1 and demonstrate that the sensitivity of the assay is high and can measure Factor I down to approximately 0.26 µg/ml or 15 ng in 50 µl. The results of the measurement of Factor I in three different normal sera are summarised in Table 2. This shows that absence of conglutination occurs at titres from 1/40 to 1/160 which corresponds to a concentration of Factor I is in the region of 10-40 µg/ml which is in agreement with other published assays. The antigenic assays carried out by radial immunoassay give slightly higher values, but the suppliers were known to have some problems with their reference Factor I level.

A conglutinating assay for the third Factor I clip that cleaves iC3b into C3c and C3dg is easier to perform than the assays looking at the earlier clips and the cell intermediate used is far more stable. The sensitivity is entirely comparable.

5. Acknowledgements

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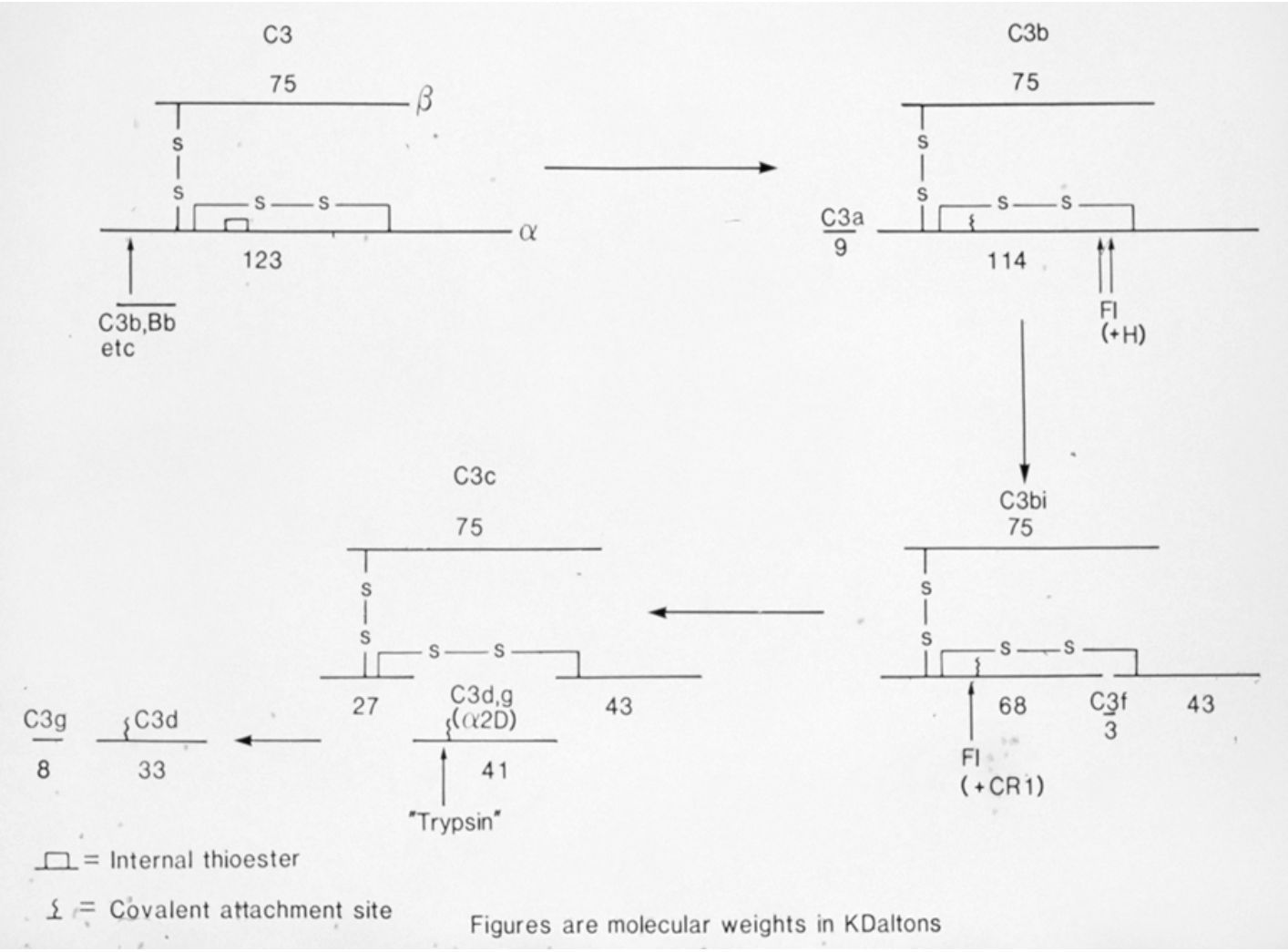

Figure 1 C3 breakdown