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An In Vitro Model of Complement Dysregulation

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wellcome

Objectives of Project

The ultimate objective of my project is to show that the antibody '**IIB6**' can be used as a model in Complement regulatory diseases. I aim to achieve this through these steps:

1. Purify the antibody 'MBI5' to use as a control for quality control checks of IIB6, and immunoglobulin characterisation.
2. Show that IIB6 binds to **Factor H** and interferes with its usual binding using Western blotting.
3. Show that IIB6 binding abnormally activates the complement system, and effects Human serum in a way that mimics disease states.

Introduction

Complement is a part of the Immune System that is responsible for protecting the body against harmful pathogens and for the surveilling and clearing of cells that die naturally. The **alternative pathway** of the complement system plays an integral role in this cell maintenance. It constantly produces proteins (**C3b**) which cause the destruction of cells, but it also produces protective proteins which defend healthy cells from being destroyed. One of these protective proteins is termed **Factor H** which controls complement in two ways: degrading C3b (into iC3b), and preventing it's further production by stopping the positive feedback loop that amplifies it (***fig.1***).

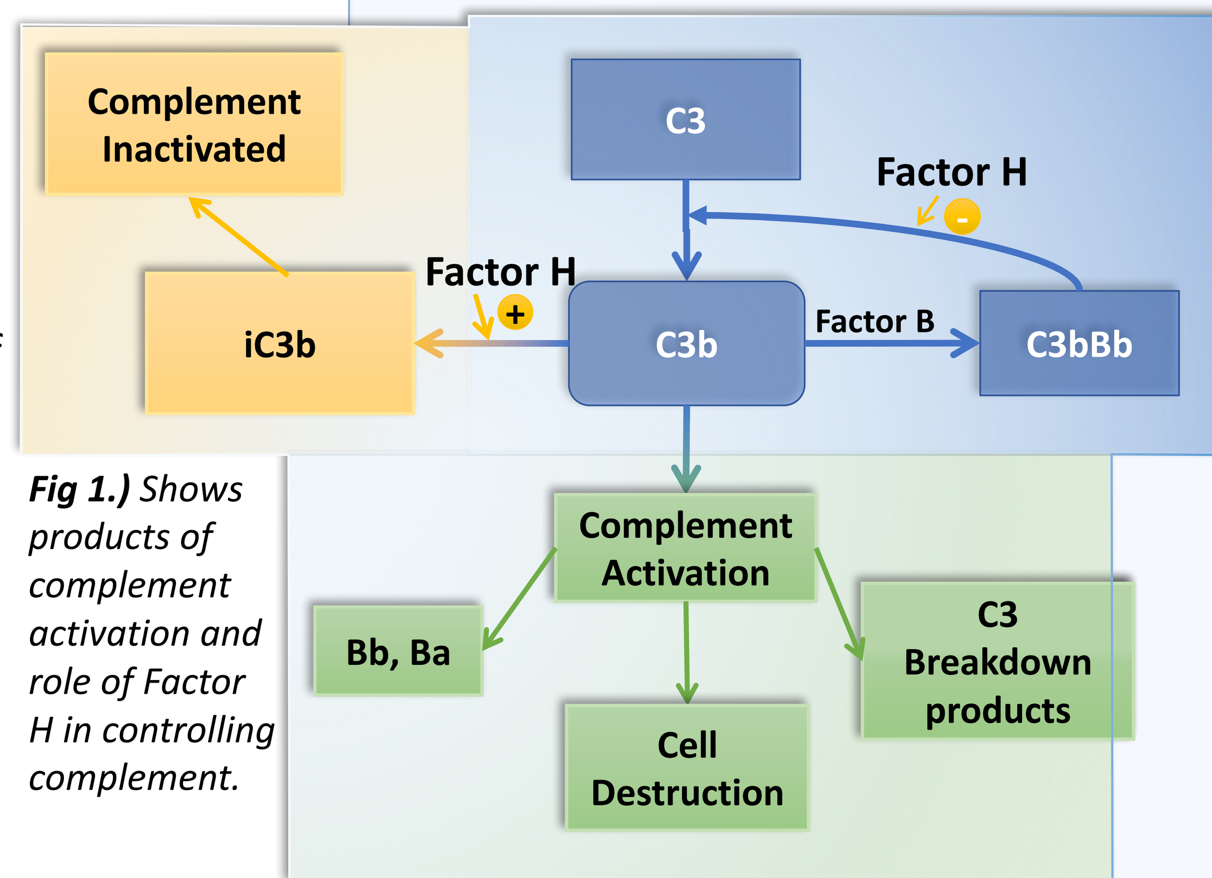


Fig 1.) *Shows products of complement activation and role of Factor H in controlling complement.*

Because the alternative pathway has the ability to spontaneously produce proteins that can damage our own cells, it is highly important that control of the system is properly maintained. One of the ways control can go wrong is by the production of **autoantibodies** which mistakenly target Factor H and cause abnormal activation of the complement system. This can manifest in severe diseases – such as Age-related macular degeneration, C3 Glomerulopathies, and Atypical haemolytic anaemia.

The ability for us to model these diseases is integral to increasing our understanding of them, and also in producing new drugs which can be used in therapy. In my project, I set out to characterise the 'artificial' autoantibody 'IIB6'. This antibody was produced by researchers in Newcastle, and was thought to bind to Factor H in a way similar to in diseases. By characterising this antibody and its effect in blood, I hoped to determine if it would make a suitable model for complement disease.

Results and Discussion

1 Purification of MBI5 and Quality control and Characterisation of IIB6

Fig.2) *AKTA pure™ chromatography machine, used for affinity purification of MBI5 from cell supernatant*

- I purified the antibody MBI5 from cell supernatant using AKTA pure™ protein G affinity chromatography ***(fig.2).***
- MBI5 was then run using SDS-PAGE and showed a strong band at 55kDa.
- Purified IIB6 was then also run, which showed a strong band at 75kDa ***(fig.3)***.

MBI5 was used as an IgG control to quality check and characterize IIB6. The differing bands showed that IIB6 was not an IgG, but an IgM. This difference greatly impacted all future experiments where antibody binding was important.

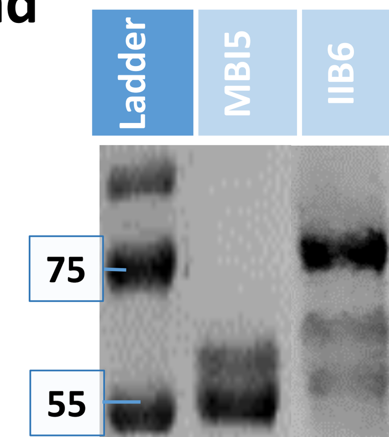


Fig.3) *SDS-PAGE of MBI5 and IIB6 with a ladder marking molecular weight in kDa.*

2 IIB6 specifically binds Factor H

- Using a western blot of IIB6 against Normal Human Serum (NHS) and pure Factor H (FH), a single band at 150kDa was found in both ***(fig.4)***.
- The 150kDa band represents the binding of IIB6 to FH.

The single band at 150kDa in NHS showed that IIB6 binds specifically to FH, which suggests that any effect it has on complement is through FH. With knowledge of the important role that FH has in regulating complement[1], it can be inferred that IIB6 potentially blocking its action would cause abnormal complement activation.

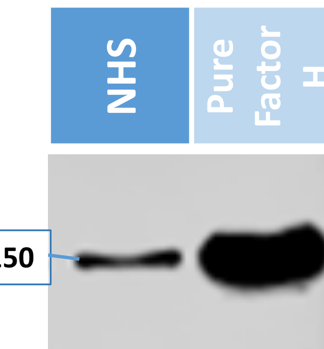


Fig.4) *Western blot of IIB6 against NHS and Pure Factor H. MW in kDa.*

3 IIB6 abnormally activates the complement system

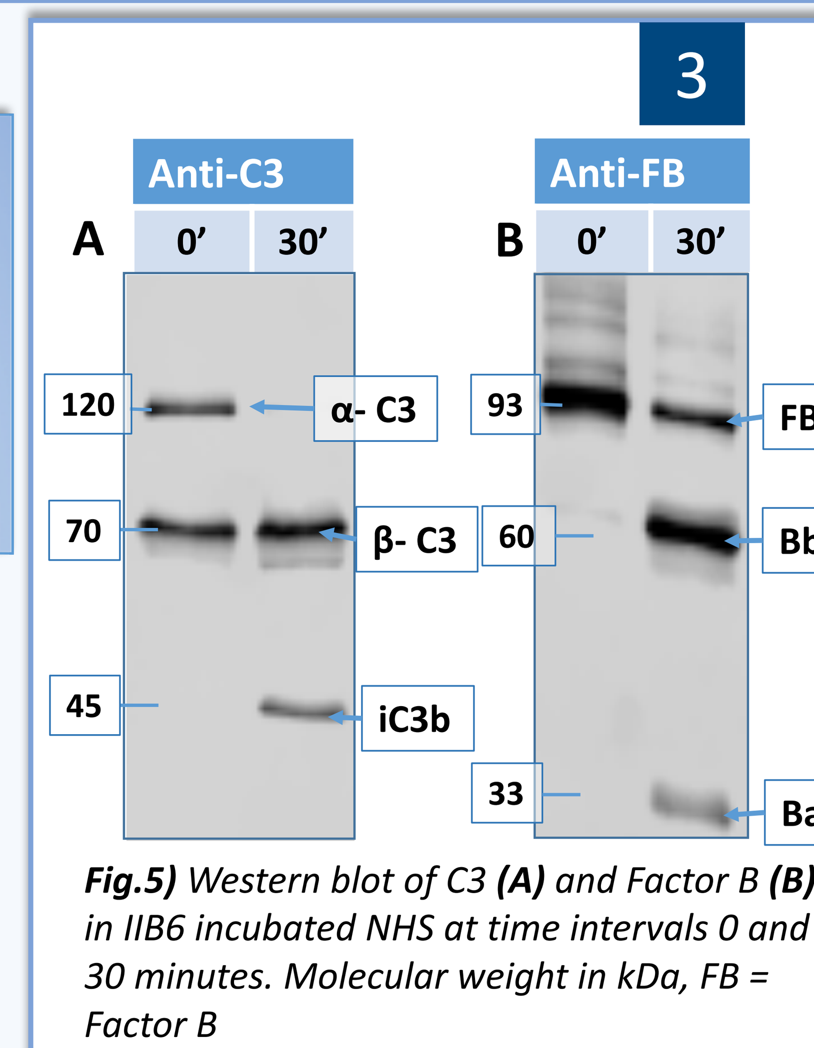
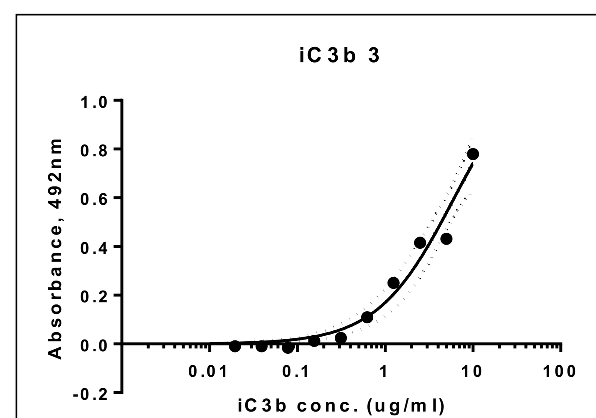


Fig.5) *Western blot of C3* ***(A)*** *and Factor B* ***(B)*** *in IIB6 incubated NHS at time intervals 0 and 30 minutes. Molecular weight in kDa, FB = Factor B*

Using a Western blot for C3 and Factor B (FB), I investigated the impact of IIB6 on alternative pathway activation.

- NHS was incubated with IIB6 for 30 minutes, with samples taken before and after. EGTA was added to serum to prevent classical pathway complement activation.
- The anti-C3 blot showed the consumption of C3 and production of the activation product iC3b ***(fig. 5A)***.
- The FB blot showed production of the two activation products, Ba and Bb ***(Fig. 5B)***.

To support the data from the western blot, an anti-iC3b ELISA was used. As expected, IIB6 incubated serum showed greatly increased concentration of iC3b than in NHS, or where complement was completely inactivated with EDTA ***(fig. 6)***. Both data together showed that activation products of complement are produced on incubation with IIB6, so suggesting the abnormally activation of complement as seen in disease states.

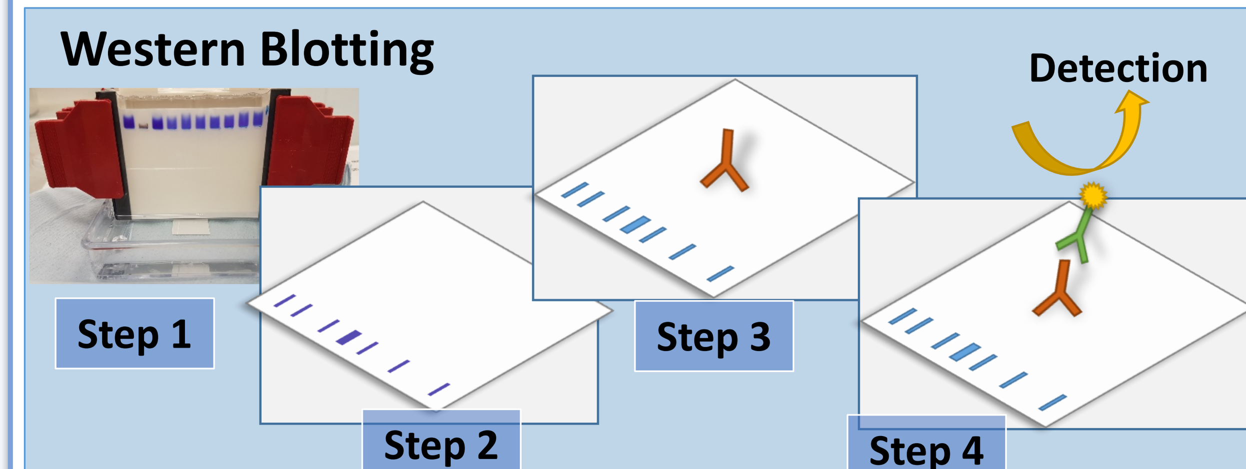


iC3b	IIB6 (µg/ml)	No IIB6 (µg/ml)	EDTA (µg/ml)
Elisa 1	460.43	258.93	211.53
Elisa 2	589.39	116.52	107.79
Elisa 3	421.58	144.69	162.18
Mean	490.46	173.38	160.50

Fig.6) *Standard Curve (top) of known iC3b concentration with interpolated values (bottom) of selected sample values.*

Methods

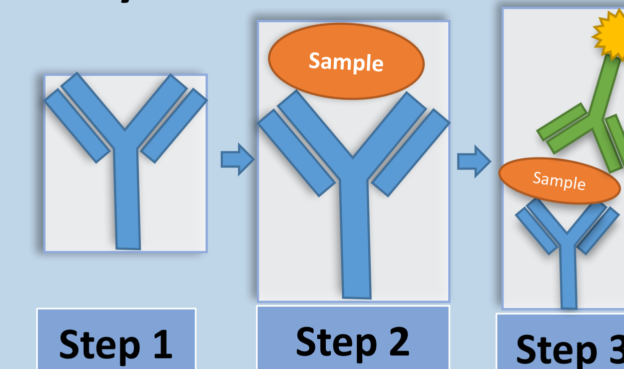
Western Blotting



Western blots were carried out to investigate IIB6's binding, and also to test for complement activation products.

1. Samples were run on SDS-page to separate the proteins
2. Separated proteins were transferred onto chromatography membranes
3. IIB6 or anti-C3/FB antibodies were incubated on the membrane, binding the sample.
4. Secondary antibodies with attached detector molecules with binding specific to the primary antibody were added to detect binding .

ELISA – Enzyme-linked Immunosorbent Assay



An iC3b ELISA was used to detect the change in production of iC3b in IIB6 incubated serum.

1. An iC3b specific capture antibody was deposited in a 96 well plate
2. IIB6 incubated and normal serum were added in duplicate to the plate, and run in a 1:2 dilution series across the plate.
3. A detector antibody was added to the plate, which also binds the sample. Analysis of detector antibody concentration can then be done.

Conclusions

From my project, I have begun to show how IIB6 can be used as a model for complement disease:

- Using affinity chromatography I purified an antibody used to identify and quality control IIB6 as an IgM.
- Through Western blotting I demonstrated the Factor H binding capabilities of IIB6 in serum.
- By Western blot and ELISA, I showed that when added to serum, IIB6 causes the production of complement activation products – mimicking the conditions seen in disease states.

Through future investigations, haemolysis assays and fluid phase co-factor assays could be used to further show that IIB6 models diseases.

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References: 1.) R.B. Pouw, D.W. Vredevoogd, T.W. Kuijpers, D. Wouters,. Of mice and men: The factor H protein family and complement regulation, Molecular Immunology, 2015;67(1): 12-20