Is complement C3 a valuable marker for the activation of the complement cascade?

P. Pitsikas, S. Cotton, A. Marin, S. Martin, H. Harouchi and J. Fontaine

charles river

1 ABSTRACT

Activation of the complement pathway involves the cleavage of Complement Component 3 (also called Complement C3) by the C3 convertase to generate the C3a and C3b split products. Following repeated or chronic complement activation, decreases in Complement C3 concentrations are observed. Complement C3 can therefore be used as a marker of repeated or chronic complement activation. Two different methods were compared when developing an assay for the quantitation of Complement C3. Despite variable levels in samples from normal or untreated animals, a proof of concept was achieved. Each stimulated sample displayed decreases in Complement C3 following activation.

In the absence of commercially available kits for the detection of non-human primate (NHP) Complement C3, one ELISA kit and one bead-based assay targeting the human protein were tested for the quantitation of NHP Complement C3. NHP serum samples were stimulated for 1 hour with different concentrations of Zymosan (from 0.067 to 3.35 mg/mL). As a reference, human plasma samples were also included, and stimulated with different concentrations of Zymosan. Unstimulated samples were incubated with PBS pH7.2 as a negative control. Unstimulated and stimulated samples were tested for Complement C3 using both methods.

The baseline variability in healthy NHPs or untreated samples was evaluated in NHPs treated with potential complement activators. Group-wise comparisons were performed, and in parallel each stimulated sample was compared to its unstimulated (vehicle) counterpart.

Zymosan is known to induce activation of the complement alternative pathway. As such, stimulation with Zymosan is expected to induce a decrease in Complement C3 concentrations. The ELISA method did not detect substantial, expected decreases, and therefore was deemed not recommended for sample analysis, where less subtle decreases may occur. The bead-based method showed a 4-fold decrease in C3 concentrations following stimulation by Zymosan in NHP serum, and 80 to 100-fold decreases in Human plasma.

A high extent of variability is observed in NHP samples, which is an obstacle to group-wise comparisons based on the presence or absence of stimulation. Indeed, the variability observed hides stimulation/treatment related decreases. However, when each animal is evaluated over time, or each sample is compared to its unstimulated counterpart, complement activation is shown using the commercially available bead-based assay.

In the absence of a well established method to detect Complement C3 in NHP, a proof of concept was established for a commercially available bead-based kit targeting human Complement C3 used to measure total C3 in NHP samples. It is evidenced by the decrease in complement C3 concentrations in human and NHP samples when stimulated with known complement activators.

In NHP samples, a high extent of variability is seen at baseline, and therefore each stimulated sample needs to be compared to its unstimulated counterpart. As such, an individual animal or subject approach for comparison of C3 data is preferable to commonly used group-wise comparisons.

Decreases in Complement C3 in response to certain drug types, such as anti-sense oligonucleotides, have been well documented. In addition, the central role of Complement C3 in the complement system and its involvement in all activation pathways, makes it a versatile and valuable marker of complement activation.

2 METHODOLOGY

One ELISA and one bead-based assay kit were tested for analytical performance and proof of concept. The following parameters were assessed:

- Curve range
- Parallelism
- Endogenous Complement C3 detection range

Proof of concept was tested on NHP serum samples (population of interest) and human plasma samples (reference population):

- Stimulation for 1 hour with Zymosan (PBS used as a negative control)
- Measurement of Complement C3 in unstimulated and stimulated samples, using the ELISA and the bead-based assay

In 2 different NHP studies, Complement C3 concentrations were measured using the bead based assay, and variations over time were evaluated in individual animals treated with know complement activators.

3 RESULTS

STD	Concentration (ng/mL)	
	ELISA	Bead-based assay
STD 10		45.00
STD 9		20.00 (ULOQ)
STD 8		10.00
STD 7	500.00 (ULOQ)	5.00
STD 6	250.00	3.33
STD 5	125.00	1.11
STD 4	62.50	0.37
STD 3	31.25	0.20
STD 2	15.63	0.12 (LLOQ)
STD 1	7.81 (LLOQ)	0.04

Table 1. Comparison of the standard curve concentrations

Parallelism assessment					
ELISA kit			Bead-based assay		
Sample identification	Dilution Factor	% Recovery	Sample identification	Dilution Factor	% Recovery
Parall-1	1000	49.3	Parall-1	1600	103.17
Parall-2	2000	79.0	Parall-2	3200	111.10
Parall-3	4000	99.9	Parall-3	6400	102.53
Parall-4	8000	100.8	Parall-4	12800	83.83
Parall-5	16000	99.3	Parall-5	25600	99.37

Table 2. Comparison of the parallelism assessments. For a dilution factor to be accepted, the %Recovery had to be within 75-125%.

Method	Endogenous Complement C3 concentrations (ng/mL)
ELISA	21931.28 to 845990.59
Bead-based assay	<LLOQ to 12282.92

Table 3. Endogenous levels of Complement C3 measured with the different kits.

ID/Status	Treatment/Condition	Complement C3 Measured concentrations (ng/mL)	
		ELISA	Bead-based assay
NHP Serum lot CYN221751	CYN221751 (unstimulated)	434273.24	668.50
	Control PBS pH7.2	423672.07	551.08
	Zymosan 3.35 mg/mL	479319.03	<LLOQ
	Zymosan 0.335 mg/mL		<LLOQ
	Zymosan 0.067 mg/mL		124.77
Human Citrated Plasma lot 1007	Control PBS pH 7.2	388015.96	>ULOQ
	5.0mg/mL Zymosan	361868.81	2556.70
	0.5mg/mL Zymosan	588650.74	175.24
Human Citrated Plasma lot 1570	Control PBS pH 7.2	356794.65	37556.87
	5.0mg/mL Zymosan	431733.15	1220.64
	0.5mg/mL Zymosan	328107.08	449.62

Table 4. Measured Complement C3 concentrations in unstimulated human and NHP samples and in their stimulated counterparts.

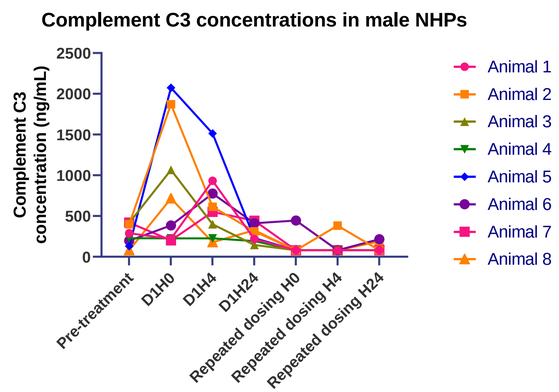

Figure 1. Endogenous levels of Complement C3 measured by the bead-based assay, in male NHPs treated with known complement activators.

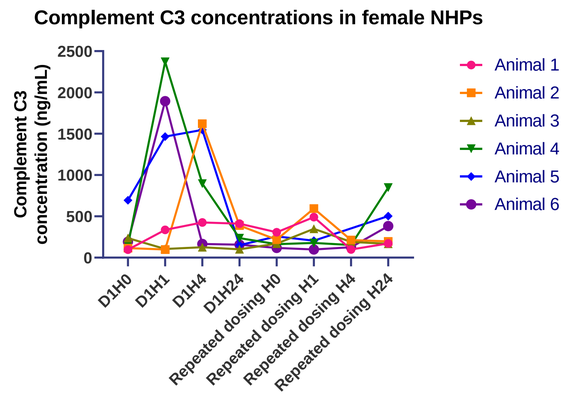

Figure 2. Endogenous levels of Complement C3 measured by the bead-based assay, in female NHPs treated with known complement activators.

4 DISCUSSION/CONCLUSION/NOVEL ASPECT

Analytical performance: In the absence of a well established method for the detection of Complement C3 in NHP, 2 commercially available kits targeting human C3 were tested and compared. Both kits showed acceptable analytical performance as depicted in **tables 1 and 2**.

Both kits were able to detect endogenous Complement C3 in baseline/normal samples, with high inter-animal variability. **Table 3**.

Proof of concept: When measured with the ELISA method, there was no evidence of Complement C3 decreases in human and NHP samples treated with Zymosan. As such, it was believed that the kit would not be able to detect a less obvious potential decrease that may be caused by varying doses of Test Items/Articles, or rule out absence of changes of Complement C3 concentrations. Substantial decreases were detected when using the bead-based assay. **Table 4**.

In study data with Complement C3 measured with the bead-based assay: A high extent of inter-animal variability was observed before treatment and in untreated animals. However, following an initial increase in concentrations, responders showed a substantial decrease in Complement C3 concentrations when subjected to repeated, long term stimulation. **Figures 1 and 2.**

In the absence of a well-established method to detect Complement C3 in NHP, a proof of concept was established for a commercially available bead-based kit targeting human Complement C3, evidenced by the substantial decrease in complement C3 concentrations in human and NHP samples when stimulated with known complement activators. It is widely agreed that the generation of samples with measurable endogenous biomarker concentrations, by *in vitro* or *in vivo* stimulation when needed, is the recommended approach in the development and validation of biomarker assays. The results presented here demonstrate that good analytical performance does not guarantee that the chosen method is suitable for the context of use. Indeed, establishment of the proof of concept is of paramount importance in the suitability of a method.

Complement activation as a safety marker, as well as decreases in Complement C3 in response to certain drug types, such as anti-sense oligonucleotides, have been well documented. Due to its involvement in all activation pathways, Complement C3 is known to play a central role in the Complement System. As such, and despite inter-animal variability at baseline, Complement C3 is considered to be a versatile and valuable marker of complement activation.