Interference from Fc-Fc Interactions in Bridging Immunogenicity Assays for IgG4 mAb Therapeutics

November, 2014

Michael Partridge Ph.D.
IgG4 Antibody Therapeutics

- Natalizumab (Tysabri): Marketed
- Pembrolizumab (Keytruda): Marketed
- Gemtuzumab ozogamicin (Mylotarg): Withdrawn
- 85 IgG4 therapeutics under investigation for clinical indications (tabs.craic.com)
- Regeneron IgG4 therapeutics
  - Multiple in various stages of non-clinical and clinical development
Unusual Features of Human IgG4

- Does not activate complement or bind Fc receptors
  - mAb therapeutic where MOA doesn’t require CDCC or ADCC
- Capable of Fab arm exchange to generate a bispecific
- Interacts via Fc under certain circumstances
Human IgG4 Fc Interactions: Background

- Human IgG4 binds to immobilized IgG via Fc
- Demonstrated by Rispens et al. with IgG immobilized on Sepharose

Human IgG4 Binds to IgG4 and Conformationally Altered IgG1 via Fc-Fc Interactions

Theo Rispens,† Pleuni Ooievaar-De Heer,† Ellen Vermeulen,† Janine Schuurman,‡ Marijn van der Neut Kolfschoten,‡ and Rob C. Aalberse†

Rispens et al. (2009) J. Imm.
Human IgG4 binds to immobilized IgG Fc in ELISA

- Low affinity, but specific to IgG4
- Underappreciated source of matrix interference

![Graph showing binding of IgG4 to IgG Fc in ELISA](image)
Interaction with immobilized IgG is an intrinsic property of all human IgG4

IgG4 reported in IgG RF may be problematic

1978

*Annals of the Rheumatic Diseases, 1978, 37, 12–17*

Antigammaglobulin (rheumatoid factor) activity of human IgG subclasses

F. Shakib and D. R. Stanworth

1987

*The Journal of Immunology*

Vol. 139, 1466–1471. No. 5, September 1, 1987

Printed in U.S.A.

The subclass distribution of human IgG rheumatoid factor

Philip L. Cohen, Robert L. Cheek, Jeffrey A. Hadler, William J. Yount, and Robert A. Eisenberg

1995

Localization of an Fc-Binding Reactivity to the Constant Region of Human IgG4

Implications for the pathogenesis of rheumatoid arthritis

Debra Jeske Zack,* Maruisz Stempniak,* Andrew L. Wong,† and Richard H. Weisbart‡
Human IgG4 may bind to Antigen-bound IgG4

- Sets of IgG1 & IgG4 variants specific for different antigens
- Only IgG4 binds to Ag-bound IgG4

**FIGURE 8.** Binding of $^{125}$I-labeled IgG4 or IgG1 (anti-Fel d 1) to IgG1 or IgG4 (anti-Bet v 1) bound to Ag-coupled Sepharose (Bet v 1). Per test, 0.6 mg of Sepharose was used (1.3 mg of Bet v 1/100 mg/Sepharose). IgG1 binding to IgG1 △, IgG1 binding to IgG4 (▲), IgG4 binding to IgG1 (■), IgG4 binding to IgG4 (percent).

IgG4 in human serum binds immobilized Fc

Biotin Anti-Human IgG4

Human IgG4

Immobilized IgG

RLU

0 (ug/mL)

20

100

500

1000

Untreated IgG

Denatured IgG
Non-Covalent Oligomers Inhibit Human IgG4 binding to Immobilized Fc

Absorbance (215 nm)

Elution Time (min)

F1 F2 F3 F4

RLU

0 50 100 150 200

10 30 100 10 30 100 10 30 100 10 30 100 10 30 100

Mouse IgG Fc Added to Human IgG4 (µg/mL)

Un. Den. F1 F2 F3 F4

Absorbance (215 nm)

Native PAGE

Biotin Anti-Human IgG4

Human IgG4

Immobilized IgG
IgG4 Fc-Fc Contacts

- IgG4 in human serum binds immobilized Fc
- Denatured IgG Fc inhibits this interaction

In Solution Fc Aggregates bind IgG4

Native Fc dimers don’t bind IgG4
Regeneron performs fully automated bioanalysis

- Allows us to analyze all phase 3 PK and ADA samples in-house
- Assays for multiple mAb and non-mAb biotherapeutics

In 2013 we began automating an ADA assay for an IgG4 mAb
- High background signal observed in negative samples
Bridging ADA Assay with IgG4 Labeled Drugs: NQC Signal Increased After Reagent Incubation

For automated sample analysis, solutions are prepared many hours in advance.

**Graph:**
- **Counts**
- **X-axis:** 0 Hours, 6 Hours
- **Y-axis:** 0 to 500
- **Legend:**
  - NQC
  - LQC (60 ng/mL)
- **Data Points:**
  - 0 Hours: NQC = 4.9 S/N, LQC = 1.7 S/N
  - 6 Hours: NQC = 4.9 S/N, LQC = 1.7 S/N

**Bar Chart:**
- Comparison between NQC and LQC signals before and after incubation.
ADA Assay: Labeled IgG4 Drug Fc-Fc Interactions

Trace Aggregates could mimic oligomeric Fc

Monomeric IgG4 labeled drugs Fc-Fc interaction?

Ruthenium labeled Drug

ADA

Biotinylated Drug

Streptavidin coated plates

Ru-Drug

1.62 %
Background Signal Increases Over Time in Bridging ADA Assay with Labeled IgG4 Drugs

Signal

Fold Signal Change

Labeled Drug Incubation Time (min) Prior to Sample Addition

5 IgG4 & 5 IgG1 labeled drugs: exogenous & endogenous targets, soluble & membrane bound targets
Excess Labeled Drug Co-incubated in Multiple Steps

Fc-Fc interactions could form in multiple steps thereby generating signal
Assay Sensitivity Decreases with Labeled Drug Incubation Time

- May impact detection of low titer positive samples
Labeled IgG4 Drugs: Increasing Signal with Plate Order

**Signal**

- **Counts**
  - Plate 1
  - **p<0.0001**
  - Plate 2
  - **p<0.0001**
  - Plate 3

- **X = NQC**

**Signal to background**

- **S/N**
  - Plate 1
  - NS
  - Plate 2
  - NS
  - Plate 3

**Screening CP Exercises: Plate Order**

- **No Effect on Screening Cut Point Determination**

**Student’s t-test (NS = not significant)**
Labeled IgG1 Drugs: No Change in Signal with Plate Order

Screening CP Exercises: Plate Order

➢ No increase in assay signal with IgG1 drugs

NS = not significant
Confirmation Assay

Without Drug

Fc-Fc contacts generate signal

With Drug

Unlabeled drug competes for Fc-Fc contacts, reducing signal
Confirmation Assay with IgG4 Labeled Drug #1

%Inhibition dependent on reagent prep. time

**p<0.001, Student’s t-test**
Confirmation Assay with IgG4 Labeled Drug #2

- %Inhibition increases with reagent prep. time

%Inhibition:
- 6 mins = 10%
- 37 mins = 27%

Counts

Without Drug

With Drug

Without Drug

With Drug

6 minutes

37 minutes

➤ %Inhibition increases with reagent prep. time
Confirmation Assay with IgG1 Labeled Drug

%Inhibition constant with reagent prep. time

%Inhibition
10 mins = 5%
45 mins = 0%

 Counts

 Without Drug
 With Drug
 Without Drug
 With Drug

10 minutes
45 minutes
Confirmation Assay: Reagent Incubation

- Signal increase greater without excess unlabeled drug
- Longer reagent incubation time = larger %Inhibition
- Reagent incubation time needs to be controlled
  - Confirmation cut point determination
  - Confirmation assay

![Graph showing signal increase over time with and without drug](image-url)
Impact on Confirmation Assay Also Observed in Plate Order

Even plate order can impact %Inhibition in confirmation cut point determination

**Student’s t-test**
For IgG4 Drugs, Plate Run Order Impacts %Inhibition in Confirmation Cut Point Determination

Drug 1: plate 3 has a statistically higher %Inhibition.

Drugs 2 and 3: plate order effect is not significant, but close.

*ANOVA (NS = not significant)

<table>
<thead>
<tr>
<th>%Inhibition Plate Order: IgG4 Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug 1</strong></td>
</tr>
<tr>
<td>*p=0.042 1&amp;2&lt;3</td>
</tr>
<tr>
<td><strong>Drug 2</strong></td>
</tr>
<tr>
<td>p=0.052 NS</td>
</tr>
<tr>
<td><strong>Drug 3</strong></td>
</tr>
<tr>
<td>p=0.066 NS</td>
</tr>
<tr>
<td><strong>Drug 4</strong></td>
</tr>
<tr>
<td>p=0.876 NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>%Inhibition Plate Order: IgG1 Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug 5</strong></td>
</tr>
<tr>
<td>p=0.29 NS</td>
</tr>
<tr>
<td><strong>Drug 6</strong></td>
</tr>
<tr>
<td>p=0.61 NS</td>
</tr>
<tr>
<td><strong>Drug 7</strong></td>
</tr>
<tr>
<td>p=0.326 NS</td>
</tr>
</tbody>
</table>
How Does This Affect Confirmation Cut Point?

- 2 analysts, 2 days, 54 individuals +/- drug
- 6-10 mins vs 20 minutes reagent incubation

<table>
<thead>
<tr>
<th>Reagent Incubation</th>
<th>Estimated</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-10 Mins</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>20 Mins</td>
<td>27</td>
<td>22</td>
</tr>
</tbody>
</table>

3% difference is within precision limits of the assay

*FP Rate = 0.1 %
**IgG4 Drugs**

Solutions: 2X Tris (acid Rx)  
4X Bio-Drug  
4X Ru-Drug  

**Screen**: Combine three solutions 2:1:1 before adding to acidified sample  

**Confirmation**: Divide 2X Tris, add unlabeled drug to one. Combine three solutions 2:1:1 before adding to acidified sample
Separating Bio-Drug & Ru-Drug Solutions Reduces Negative Control Signal

- Combined Bio-Drug & Ru-Drug
- Separate Bio-Drug & Ru-Drug Containing Tris
- Separate Tris, Bio-Drug, Ru-Drug &

Counts

- 0 Hours
- 6 Hours
Excess Labeled Drug Co-incubated in Sample: Long Incubations

1) Reagent Preparation (Automation)
2) Sample Incubation (O/N incubation)
3) Assay Plate Incubation
4) Read Buffer
Overnight Sample Incubation in Serum Does Not Generate High Signal

Counts

Without Serum

With Serum (3.3%)

Sample Plate Incubation Time

Bio & Ru-IgG4

Bio & Ru-IgG1
Serum Reduces IgG4 Fc Interactions

- Human IgG4 ≈ 100 - 500 µg/mL in serum
  ≈ 3 - 15 µg/mL in 3.3% serum

- Monkey (and rat) serum also block signal
IgG4 Fc-Fc Interacts with Other IgGs
IgG1:IgG4 Pairing

Labeled Drug Incubation Time (mins)

- Bio & Ru-IgG4
- Bio & Ru-IgG1
- Bio-IgG4 & Ru-IgG1
- Bio-IgG1 & Ru-IgG4
Conclusions

- Fc interactions between human IgG4 labeled drugs can cause increased signal over time in bridging assays
- Decreases assay sensitivity – Detection of low titer antibodies
- Major impact - Confirmation assay
- Prepare labeled drug solutions separately
- Cons of bridging ADA assays
- Fc Interference in specific disease indications
- Affects IgG4 formulations?
Acknowledgements

Elif Karayusuf
Olena Dziadiv
Onson Luong
John Garlits
Thanoja Sirimanne
Jihua Chen
Bob Dreyer
Ganga Dhulipala
Erica Pyles
Enoch Shum
Uma Vijayam

Giane Sumner
Manoj Rajadhyaksha
Al Torri