Antibody investigations with immobilised HIV antigen and Fc receptors.

Paul Rogers
We are a group of around 28 in the Department of Medicine, Division of Infectious Disease based at St Mary’s Hospital in Paddington.

Group Leader Professor Robin Shattock

- Particular interests:
  - HIV vaccine development
  - Mucosal immunity
  - Microbicides
I apologise in advance for the vagueness of some of the descriptions. Most of these results are part of larger studies submitted for review prior to publication.

This being a workshop I have concentrated on methodology and want to share experiences that have arisen during our studies.

- HIV vaccine studies - ARG2 biosensors
- Fcy receptor binding - Ni-NTA biosensors
**HIV vaccine studies**

- We are interested in the response to vaccination in terms of antibody kinetics in the serum and the mucosa.

- Currently investigating a DNA vaccine in an in vivo model. The DNA codes for an HIV surface protein.

- Three groups of 6 animals
  - Grp 1 - 3xDNA Prime / Protein Boost / Protein Boost
  - Grp 2 - DNA Prime alone
  - Grp 3 - Protein Boost / Protein Boost

- Blood taken 1 week post DNA priming, post first and post second protein boost
Why a BLItz approach to kinetics?

- **Small volumes** - You don’t get a lot of volume out of a mouse or rabbit - the BLItz can measure 5μl.

- **Denaturing ELISA** - Hardly reproduces a physiological situation

- **Rapid results** - Amenable to scale-up (Octet)

**Issues**
- Serum contains primarily IgA and IgG (75%) (4/2 binding sites respectively).
- Mucosal samples primarily IgA (85%).
- Not strictly possible to determine $K_a$ and $KD$ from a mixture.
BLItz Strategy

- Covalently immobilise HIV antigen to ARG2 biosensors with amide linkage.

- Take blood from vaccinated animals and spin down.

- Dilute serum to 5mg/ml total protein.

- Assay Buffer – DPBS + 100μg/ml BSA + 0.01% Tween-20.
We used the Basic Kinetics program to investigate optimal NHS/EDC concentration (ratio 1:4) and diluent.

The aim was to maximise the mass bound to the biosensor.

10mM Na-Acetate is more effective than water. We selected 50mM NHS/200mM EDC.
Biosensors activated as previously determined.

No increase >100μg/ml

Immobilisation pH sensitive – pH4> pH6.

Experiment further with more pH values.
• Biosensors activated as previously determined.

• Balance maximum binding with physiological pH
Protocol

• **Biosensor preparation**
  1. Hydrate ARG2 biosensors >10min.
  2. Activate biosensors with 50mM NHS/ 200mM EDC for 8min.
  3. Wash
  4. Bind protein at 100μg/ml in 10mM Na-Acetate (pH 4.5) for 15min.
  5. Wash in 100mM Tris-HCl
  6. Cap biosensor in 1M Ethylene diamine

• **Assay**
  • 30 secs Baseline
  • 300 secs Association
  • 600 secs Dissociation

• **Single use of Biosensor**
Second bleed – post first protein boost

Graph showing the signal minus baseline over time for different groups (Grp 1-3) with different samples (#1-6). The x-axis represents time in seconds, ranging from 0 to 900, while the y-axis represents signal minus baseline, ranging from -0.5 to 1.9.
• The wavelength shifts following protein boost for Group 1 (DNA/Protein) but not for the DNA and Protein control groups.
• Using the 25% and 75% signal intensities to calculate average rates of change, the serum at the second bleed (4.21 x 10^{-3}nms^{-1}) had a 1.93-fold and the serum from the final bleed (5.99 x 10^{-3} nms^{-1}) a 3.25-fold higher rate of increase than the serum from the first bleed (1.84 x 10^{-3}nms^{-1})

• The $K_d$ is reduced in Group 1 between Bleed 1 and Bleed 2.
Issues

- The high protein concentration in the samples results in a matrix effect – as can be seen from the spikes in signal when changing from Buffer to sample.

- Diluting the sample further will reduce the signal in the earliest bleeds.

- Judgement must be employed when distinguishing strong binders from non-binders.

- In common with other methods of measuring affinity etc., low levels of binding are difficult to analyse.

- Further experiments with more samples are justified.
**Fcγ receptor binding**

- Our group is interested in the way that Fcγ receptors present on NK cells and macrophages interact with virus-bound IgG and we wanted to measure the binding kinetics.

- Our initial experiments used an SPR machine (GMC)

- Problems included:
  - Expense and time to coat chips.
  - Non-regeneration of Fcγ receptors.

- BLItz
  - Anti-receptor IgG bound to Prot-A biosensors no good for measuring receptor-IgG interaction.

  - Covalent immobilisation of receptor onto ARG2 biosensors not successful.

  - Recombinant receptors have a C-terminal 6-His tag.
Ni-NTA Biosensor coating with r-Fcy receptor

- The more receptor coated, the higher the signal.

- After a few more optimisations, 10μg/ml receptor and an increased vibration rate was selected as a balance between signal and economy.
DPBS/BSA buffer evaluation

- **DPBS/BSA contains 100μg/ml BSA plus 0.01% Tween-20**

$R_{\text{max}} = 0.7$
HBS-EP buffer evaluation

- 10mM HEPES, 150mM NaCl, 3mM EDTA, 0.005% Tween-20 (pH 7.4)

- A higher and more stable binding, the HBS-EP buffer was employed for samples
Ni-NTA biosensor regeneration

- Biosensor stripped with three 30 second cycles of 50mM Glycine (pH 2.5) and buffer.
- Regenerated with 60secs 10mM NiCl$_2$
- Stable for >6 regenerations
Parameters

• **SOP**
  • To ensure consistency and comparability between runs.

• **Pre-conditioning.**

• **Acceptance Criteria**
  • I use the coating step to check consistency within and between runs.
  • If the $R_{\text{max}}$ is within the 95%CI of the last 10 runs – acceptable.
  • At least 3 usable dilutions.

• **Analysis**
  • Take off Baseline.
  • Global fit.
  • $K_a$ and $K_d$ errors <20%.
SOP and acceptance criteria

- The red line occurred when a larger volume of liquid was placed into the drop.
- The acceptance criteria rules out the lower and upper measurements.
• When manually loading sample onto the drop, be careful not to introduce air bubbles
<table>
<thead>
<tr>
<th>Index</th>
<th>Sample ID</th>
<th>Conc. (nM)</th>
<th>KD (M)</th>
<th>ka (1/Ms)</th>
<th>ka Error</th>
<th>kd (1/s)</th>
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<td>7.522</td>
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HIV-1 bound IgG vs high affinity Fc receptor

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<td>3</td>
<td>2.5μg/ml IgG + 100pg/ml HIV</td>
<td>17.1</td>
<td>2.23E-08</td>
<td>3.79E+04</td>
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### α-HIV IgG alone vs medium affinity Fc receptor

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## HIV-1 bound IgG vs medium affinity Fc receptor

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### α-HIV IgG alone vs low affinity Fc receptor

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<td>6.44E+04</td>
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<td>3</td>
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<tr>
<td>4</td>
<td>5μg/ml IgG</td>
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## HIV-1 bound IgG vs low affinity Fc receptor

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<tr>
<td>2</td>
<td>2.5μg/ml IgG + 400pg/ml HIV</td>
<td>17.1</td>
<td>&lt;1e-12</td>
<td>5.57E+03</td>
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<td>-1.874</td>
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<td>4</td>
<td>10μg/ml IgG + 200pg/ml HIV</td>
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<td>5.57E+03</td>
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<td>5</td>
<td>20μg/ml IgG + 400pg/ml HIV</td>
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<td>&lt;1e-12</td>
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<td>0.1606</td>
<td>0.9887</td>
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# Summary of Fcγ results

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<th>KD (nM)</th>
<th>Ka (1/Ms)</th>
<th>Ka Error</th>
<th>Kd (1/s)</th>
<th>Kd Error</th>
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<tbody>
<tr>
<td>High Affinity Fc Receptor - IgG alone</td>
<td>68.5</td>
<td>1.75E+04</td>
<td>3.07E+03</td>
<td>1.20E-03</td>
<td>2.47E-05</td>
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<td>High Affinity Fc Receptor - HIV-bound IgG</td>
<td>22.3</td>
<td>3.79E+04</td>
<td>3.18E+03</td>
<td>0.843E-03</td>
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<td>Medium Affinity Fc Receptor - IgG alone</td>
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<td>6.31E-05</td>
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<td>Medium Affinity Fc Receptor - HIV-bound IgG</td>
<td>32.7</td>
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\[ KD = \frac{K_d}{K_a} \quad \text{The lower the} \ KD, \ \text{the stronger the binding.} \]

You want a low \( K_d \) and a high \( K_a \) for minimum \( KD \)

- The high-affinity receptor has a slightly lower \( KD \) for IgG alone than the medium-affinity receptor.

- The \( K_a \) of high and medium-affinity receptors is increased for virus-bound antibodies, \( K_d \) is similar resulting in lower \( KD \).
Issues

- When calculating values for antibody bound to virus, how to take account of the increasing mass?

- Judgement must be employed when distinguishing strong binders from non-binders.

- Experiments continue to confirm the results and look at different HIV strains.
Review

- **BLItz Software** - no stats, exporting tricky, can’t name assay stages.
- **Versatile**  – Other projects are ongoing using different binding chemistries.
- **Robust**  - No liquid handling.
- **Compact**  - Space can be at a premium.
Acknowledgements

• Dr Jamie Mann
• Prof Robin Shattock