Neutralizing anti-drug antibodies

Emerging Trends and Clinical Impact

A. Kromminga
What is immunogenicity?

The ability of a substance (e.g. antigen or vaccine) to elicit an immune response

Innate immunity

- Cell mediated cytotoxicity
- ADCC (antibody dependent cellular cytotoxicity)
- CDC (complement dependent cytotoxicity)

Antigen specific immune responses

- T cell activation
- Cytokine storm
- Hypersensitivity

Anti-drug antibodies (ADA)
Complexity of the immune response
FDA says:

Because of the size of some clinical trials and the necessity of testing patient samples at several time-points, FDA recommends a multi-tiered approach to the testing of patient samples. 

Neutralizing antibodies (NAB) are generally of more concern than binding antibodies (BAB) that are not neutralizing, but both may have clinical consequences.
Questions

Why are neutralizing antibodies (NAB) of more concern than binding antibodies (BAB) that are not neutralizing if both may have clinical consequences?

Do NAB assays more realistically reflect the situation in the body?
FDA says:

Generally, bioassays have significant variability and a limited dynamic range for their activity curves. Such problems can make development and validation of neutralization assays difficult and FDA understands such difficulties. Nonetheless, we will recommend such assays because they are critical to understanding the importance of patient immune responses to therapeutic proteins.
ADA Analysis

Screening ADA Assays
- ELISA
- ECL
- DELFIA
- Gyros
- FEIA
- RIPA
- SPR

NAB assays

Cell based Assays
- Cell proliferation
- Biomarker
- Gene expression
- Gene reporter
- ADCC
- CDC

Non-cell based Assays
- CLBA
- SPR
## Purpose of ADA vs NAB assay

### Screening ADA assays
- Analytical sensitivity: < 500 ng/ml
- Clinical sensitivity: 100%
- Clinical specificity: 95%
- Drug interference: n.d.

### NAB assays
- Analytical sensitivity: n.d.
- Clinical sensitivity: n.d.
- Clinical specificity: 100%
- Drug interference: n.d.
FDA immunogenicity guideline, 2009:

• Generally FDA considers that bioassays are more reflective of the in vivo situation and are recommended.

• For NAB assays, the bioassay should be related to product mechanism of action, otherwise the assay will not be informative as to the effect of NAB on clinical results.

• The development and validation of neutralization assays may be difficult and FDA understands such difficulties. Nonetheless, we will recommend such assays because they are critical to understanding the importance of patient immune responses to therapeutic proteins.
Cell-based versus non-cell based NAB detection

**EMA immunogenicity guideline, EMEA/CHMP/BMWP/14327/2006:**

- If neutralising cell-based assays are not feasible/available competitive ligand binding assays or alternatives may be suitable.
- However, when these are used, it must be demonstrated that they reflect neutralizing capacity/potential in an appropriate manner.
NAB analysis
by a competitive ligand binding assay
## Validation data of CLB

<table>
<thead>
<tr>
<th>NAB</th>
<th>Run1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Mean</th>
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<tbody>
<tr>
<td></td>
<td>Signal</td>
<td>%CV</td>
<td>Signal</td>
<td>%CV</td>
<td>Signal</td>
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<td>blank</td>
<td>0.012</td>
<td>-</td>
<td>0.015</td>
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</table>
Requirements for cellular assays

- Suitable cell line
- Linearity
- Interference
- Cut point
- Sensitivity
- Specificity
- Precision
- Robustness
- Ruggedness
Example: Erythropoietin

- recombinant human protein drug with a non-redundant endogenous counterpart
- used for the treatment of renal and non-renal anemia
Antibodies against EPO

1. Pure red cell aplasia (PRCA) after initial successful erythropoietin therapy
   - Progressive, transfusion-dependent anemia
   - Almost total lost of erythroid progenitor cells with normal BM

2. Antibodies against erythropoietin

3. No endogenous erythropoietin detectable
NAB-EPO Detection

Based on the inhibition of drug-specific proliferation in the presence of ADA.
There is no interaction with IL-3 leading to a significant change of inhibitory effect of anti-EPO antibodies. Average IL-3 level in normal healthy subjects is 27 pg/ml.
# Sensitivity/LLOD

<table>
<thead>
<tr>
<th>Monkey anti-EPO (ng/ml)</th>
<th>%-Inhibition</th>
<th></th>
<th></th>
<th></th>
<th>SD</th>
<th>%CV</th>
<th>AC</th>
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</thead>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>mean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>50.1</td>
<td>52.2</td>
<td>55.3</td>
<td>52.5</td>
<td>2.1</td>
<td>4.1</td>
<td>≤ 30%</td>
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<tr>
<td>100</td>
<td>71.9</td>
<td>70.6</td>
<td>71.9</td>
<td>71.5</td>
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<td>6.4</td>
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<td>17.1</td>
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<tr>
<td>12.5</td>
<td>7.3</td>
<td>6.0</td>
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<td>3.0</td>
<td>34.8</td>
<td>≤ 30%</td>
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<tr>
<td>6.25</td>
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<td>0.3</td>
<td>7.3</td>
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<td>67.1</td>
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<td>3.13</td>
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<td>6.2</td>
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<td>19.0</td>
<td>≤ 30%</td>
</tr>
<tr>
<td>1.55</td>
<td>-0.1</td>
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<td>94.9</td>
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</tr>
<tr>
<td>0</td>
<td>-5.2</td>
<td>2.4</td>
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<td>0.0</td>
<td>3.7</td>
<td>-</td>
<td>≤ 30%</td>
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## Precision

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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>mean</th>
<th>SD</th>
<th>%CV</th>
<th>AC</th>
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</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-Assay</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>98.6</td>
<td>95.1</td>
<td>100.8</td>
<td>98.0</td>
<td>98.1</td>
<td>2.6</td>
<td>2.1</td>
<td>≤ 30%</td>
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<tr>
<td>Inter-Assay</td>
<td></td>
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<td></td>
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<td></td>
</tr>
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<td>37.2</td>
<td>35.5</td>
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<td>37.1</td>
<td>36.9</td>
<td>0.8</td>
<td>2.6</td>
<td>≤ 30%</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th></th>
<th></th>
<th></th>
<th>mean</th>
<th>SD</th>
<th>%CV</th>
<th>AC</th>
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<tbody>
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<td>98</td>
<td>100</td>
<td>99</td>
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<td>99</td>
<td>0.9</td>
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<td>≤ 30%</td>
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<tr>
<td></td>
<td>46</td>
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<td>37</td>
<td>41</td>
<td>41</td>
<td>3.7</td>
<td>9.1</td>
<td>≤ 30%</td>
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</table>
## Summary: NAB EPO

<table>
<thead>
<tr>
<th>Validation characteristics</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenging concentration of EPO</td>
<td>20 pM</td>
</tr>
<tr>
<td>Intra-assay precision</td>
<td>≤ 2 % CV</td>
</tr>
<tr>
<td>Inter-assay precision</td>
<td>≤ 9 % CV</td>
</tr>
<tr>
<td>Stability for 3 days at +2-8°C</td>
<td>≤ 8 % deviation</td>
</tr>
<tr>
<td>Stability for 3 weeks at -20°C</td>
<td>≤ 12 % deviation</td>
</tr>
<tr>
<td>Stability at ≤ -15 °C after 3 Freeze/Thaw cycles</td>
<td>≤ 11 % deviation</td>
</tr>
<tr>
<td>Stability at ≤ -70 °C after 3 Freeze/Thaw cycles</td>
<td>≤ 14 % deviation</td>
</tr>
<tr>
<td>Drug tolerance</td>
<td>250 mIU/ml</td>
</tr>
<tr>
<td>Clinical Specificity</td>
<td>100 %</td>
</tr>
<tr>
<td>Cross reactivity against IL-3</td>
<td>none</td>
</tr>
<tr>
<td>Screening cut point (% inhibition)</td>
<td>17 %</td>
</tr>
<tr>
<td>Sensitivity in 2% serum</td>
<td>25 ng/mL</td>
</tr>
<tr>
<td>Sensitivity in undiluted serum</td>
<td>1250 ng/mL</td>
</tr>
<tr>
<td>Minimum required dilution (MRD)</td>
<td>2 % serum</td>
</tr>
</tbody>
</table>
Example: FSH

- recombinant human protein drug with an endogenous counterpart
- used for the treatment of induction of ovulation/pregnancy and for the development of multiple follicles.
NAB against FSH
Hormonal treatment of cultured cells

Inhibition of hormonal effect by NAB
# NAB FSH

<table>
<thead>
<tr>
<th>Validation characteristics</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenging concentration of FSH</td>
<td>1 mIU/ml</td>
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<tr>
<td>Intra-assay precision</td>
<td>≤ 4 % CV</td>
</tr>
<tr>
<td>Inter-assay precision</td>
<td>≤ 14 % CV</td>
</tr>
<tr>
<td>Stability for 3 days at RT</td>
<td>≤ 11 % deviation</td>
</tr>
<tr>
<td>Stability for 3 days at +2-8°C</td>
<td>≤ 5 % deviation</td>
</tr>
<tr>
<td>Stability at ≤ -15 °C after 3 Freeze/Thaw cycles</td>
<td>≤ 11 % deviation</td>
</tr>
<tr>
<td>Stability at ≤ -70 °C after 3 Freeze/Thaw cycles</td>
<td>≤ 14 % deviation</td>
</tr>
<tr>
<td>Drug tolerance at 150 µg/ml</td>
<td>7.5 ng/mL</td>
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<td>Drug tolerance at 15 µg/ml</td>
<td>0.75 ng/mL</td>
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<tr>
<td>Clinical Specificity</td>
<td>99 %</td>
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<tr>
<td>Cross reactivity against LH, TSH, CGalpha</td>
<td>None</td>
</tr>
<tr>
<td>Screening cut point (% inhibition)</td>
<td>23 % inhibition</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>Minimum required dilution (MRD)</td>
<td>2 % serum</td>
</tr>
</tbody>
</table>
Example: Interferon

- recombinant human protein drug with an endogenous counterpart
- used for the treatment of
  - Multiple Sclerosis (IFN-β) and
  - Hepatitis virus infection (IFN-α)
Gene expression assay
NAB against interferon

1. IFN-beta binds to sol. IFNAR2.
2. IFN-beta activates IFNAR2, leading to the activation of IFNAR1.
3. The activated IFNAR2 complex interacts with JAK1 and STAT1/STAT2.
4. The complex moves to the cytoplasmic membrane.
5. JAK1 phosphorylates STAT1/STAT2.
6. The phosphorylated STAT1/STAT2 complex translocates to the nucleus.
7. STAT1/STAT2 binds to ISRE (interferon-stimulated response element), activating transcription of genes such as MxA, TRAIL, ISG15, LMP7, CTSS, XAF-1, and others.

Note: The diagram visualizes the signaling pathway for interferon-activated gene expression.
Gene expression assay
NAB against interferon by MxA analysis

**Positive** sample: sample signal < EC50
Example: mab
Inhibition of ADCC by anti-IS

Target cell line: SK-BR3i
MRD: 2 %
Effector cell: CD16-NK
Drug: anti-Her2/neu
Inhibition of CDC by anti-IS

Target cell line: Raji
MRD: 2%
Effector: complement (10:1)
Drug: anti-CD20
Mechanism of action via TNFα signaling

TNFα signaling pathway

Plasma membrane

Nuclear membrane

Gene Regulation

Cell death

Gene Regulation

Apoptosis

Cell death
TNFα / TNFα receptor signaling via the apoptosis pathway increases caspase 3 activity.

A TNFα blocker drug dose-responsively lowers caspase 3 activity of TNFα by blocking TNFα binding to receptors.
Bioluminescent caspase-based bioassay of TNFα blocker drug activity on TNFα signaling

- Rapidly responsive human U937 cells in provide bioassay high consistency
- Bioluminescence readout provides excellent bioassay sensitivity and dynamic range
- Fast assay (2.5 hr response)
TNFα blocker cell-based bioassay based on NF-κB luc reporter activity

TNFα / TNFα receptor signaling via the NF-κB pathway increases gene expression driven by the NF-κB response element.

A TNFα blocker drug dose-dependently lowers NF-κB driven luciferase activity of TNFα by blocking TNFα binding to receptors.
Bioluminescent NF-κB reporter gene bioassay of TNFα blocker drug activity on TNFα signaling

- Stably transfected human NF-κB HEK-293 cells provide high consistency
- Bioluminescence readout provides excellent bioassay sensitivity and dynamic range
- Fast assay (4 hr induction of NF-κB driven luciferase expression)
TNFα Resp Reporter Gene Construct

Lallemand C, et al, Tovey MG. J Immunol Methods. 2011
Anti-TNFα-NAB analysis

Lallemand C, et al, Tovey MG. J Immunol Methods. 2011
Correlation with clinical responses is usually necessary to determine the clinical relevance of both binding and neutralizing antibody responses.

FDA Guidance, 2013
Left and right hand
Rheumatoid Arthritis

- Prevalence: 1.0%
- f/m: 2.5/1
- Age: 43 (± 40)
- Chronic synovialitis
- Anti-CCP antibodies (CCP)

Nijenhuis S et al Clin Chim Acta, 2004
Targets of treatment in RA
### Case 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Geb.-Datum</th>
<th>Kasse</th>
<th>Labor-Nr./Eingang</th>
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<tr>
<td>w</td>
<td>03.07.1987</td>
<td>BMA</td>
<td>Labor Lademannbogen MfZ GmbH</td>
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<th>Einheit</th>
<th>Referenz</th>
<th>Methodik</th>
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<tbody>
<tr>
<td>AK gg. Infliximab</td>
<td>negativ</td>
<td>µg/ml</td>
<td>negativ</td>
<td>EIA</td>
</tr>
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</table>
Case 2

Slightly increased antibodies against infliximab detectable. In case of clinical signs of loss of efficacy or therapeutic non-responsiveness, consider a change of treatment.

Sonic Healthcare Labor Lademannbogen
Case 3

Increased antibodies against infliximab detectable. In case of clinical signs of loss of efficacy or therapeutic non-responsiveness, consider a change of treatment.

Sonic Healthcare Labor Lademannbogen
The measurement of antibodies against TNF-alpha inhibitors were performed using different assay formats.

Antibodies against infliximab were detectable on 08.01.2010 by both an ELISA-based method and a cell-based bioassay. These results were confirmed on 22.03.2011. The cell-based assay could not be used at that time, possibly due to circulating infliximab levels.

The same results were obtained on 19.04.2011 (positive by ACE-ELISA and not evaluable by bioassay).

In case of clinical signs of loss of efficacy or therapeutic non-responsiveness, consider a change of treatment.
• Assays for the detection of neutralizing antibodies are to be included in the cascade of immunogenicity assessment.

• Neutralizing antibodies (NAB) are generally of more concern than binding antibodies (BAB).

• The detection of NAB can be performed by cell-based assays (CBA) or by non-cell-based competitive ligand binding assays (CLBA).

• FDA prefers CBA because these more realistically reflect the in vivo situation.

• Sometime cell-based assays are more difficult and tedious to establish. Recombinant cell lines / reporter gene readouts may be an alternative for the NAB analysis if other cell-based assay are not available.

• A therapeutic ADA/NAB monitoring should be mandatory in all patients treated with Biologicals.
Future Hamburg