Principles of Antibody Engineering and Therapy

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Antibodies recognize foreign substances (antigens)

examples: bacteria
           viruses
           cancer cells
           pollen (allergies)

They have the ability to recognize millions of different antigens

Carry out “effector functions”

Examples: kill bacteria
           prevent viral attachment to cells
           neutralize toxins
           destroy cancer cells
How are they able to do all of this?

Antibodies are remarkable molecules with a division of labor.

They have variable regions that are the part of the molecule that binds antigen. There are literally millions of different possible variable regions so antibodies can recognize millions of different antigens.
How are they able to do all of this?

Antibodies are remarkable molecules with a division of labor.

They have a relatively constant region. It is this region that is responsible for carrying out the limited number of different effector functions.
Considerations when choosing or making an Ab

Specificity: epitope affinity
Determined by the Variable Region

Functional properties: Half-life
Fc receptor binding
Complement activation
Tissue penetration
Determined by the Constant Region
The Ab can be divided into different functional regions and Ab fragments have many useful properties.

However the focus of this presentation will be on intact Abs
Original Source of Antibodies Was Murine Hybridomas

Advantages: Many precisely characterized specificities
         Homogeneous
         Available in virtually unlimited quantities
         Single constant region with associated effector functions

Disadvantage: IMMUNOGENICITY
A solution was to produce chimeric Abs with the variable region from the mouse Ab joined to a human constant region.
Mouse Antibody

Variable region from mouse recognizes the same antigen
Constant region from human
human effector functions

Chimeric Antibody

QuickTime™ and a TIFF (Uncompressed) decompressor are needed to see this picture.
Chimeric Antibody

Since this antibody is mostly human it is usually not recognized as foreign

Examples in the clinic: Remicade (treat arthritis)
                    Rituxin (treat lymphoma)
The CDRs are loops extending from the variable regions so that they are easily accessible for interaction with Ag. The other amino acids in the variable region are the “framework” amino acids and provide a scaffold to maintain the CDRs in the proper orientation.

It is the CDRs that determine the binding specificity of the antibody.
It is possible to transfer the CDRs from a mouse variable region to a human variable region.

Chimeric Antibody

CDR (loop)-grafted Antibody

Recognizes the same antigen
Almost completely human
CDR (loop)-grafted Antibody

Recognizes the same antigen
Almost completely human

Examples in the clinic: Herceptin (breast cancer)
Synagisis (RSV in infants)
It is possible to immunize a mouse and obtain human Abs

The Xenomouse™ has the murine Ig loci disrupted and contains the information to make a human Ab
It is also possible to obtain specific antibodies without using an animal.

Antibody binding specificities can be expressed on the surface of bacteriophage (bacterial viruses) and selected using phage binding to antigen.

VH and VL can be obtained from either naïve or immunized animals of diverse species including man.
B cells harvested from blood

VH genes

VL genes

PCR cloning of VH and VL gene repertoires

PCR assembly and cloning into a phage display vector

Selection

1) Bind phage to antigen
2) Wash away nonbinding phage
3) Elute binding phage and amplify in E. Coli
4) Repeat 3-4 times

Production in E. coli culture

Screening

Desired scFv or Fab

Round 1

Round 2

Round 3

Round 4

Can make complete Ab
Using the Techniques of Antibody Engineering it is Possible To Produce Abs with the Desired Functional Properties

- Half-life
- Fc receptor Binding
- Complement Activation
One important question is what determines the *in vivo* persistence of antibodies

An important role for FcRn has emerged

Junghans & Anderson, PNAS 93:5512

**Selective depression of plasma IgG concentration in β2m-/mice**

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2200 ± 100</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>Mutant</td>
<td>260 ± 30</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>Ratio</td>
<td>8.4:1 ± 0.9</td>
<td>1.0:1 ± 0.2</td>
</tr>
</tbody>
</table>
FcRn Binds IgG at the CH2/CH3 Interface

Model for Role of FcRn

Junghans & Anderson, PNAS 93:5512
An example of increasing FcRn binding and half-life

Table II. Binding of OST577 Abs to human FcRnα

<table>
<thead>
<tr>
<th>OST577</th>
<th>n</th>
<th>IC_{50} (μg/ml)</th>
<th>Relative Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 WT</td>
<td>5</td>
<td>10.3 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>IgG1 T250Q</td>
<td>5</td>
<td>3.14 ± 0.86</td>
<td>3.3</td>
</tr>
<tr>
<td>IgG1 M428L</td>
<td>5</td>
<td>0.896 ± 0.304</td>
<td>11</td>
</tr>
<tr>
<td>IgG1 T250Q/M428L</td>
<td>5</td>
<td>0.351 ± 0.144</td>
<td>29</td>
</tr>
</tbody>
</table>

Table III. Binding of OST577 Abs to rhesus FcRnα

<table>
<thead>
<tr>
<th>OST577</th>
<th>n</th>
<th>IC_{50} (μg/ml)</th>
<th>Relative Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 WT</td>
<td>3</td>
<td>8.86 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>IgG1 T250Q</td>
<td>3</td>
<td>2.97 ± 0.59</td>
<td>3.0</td>
</tr>
<tr>
<td>IgG1 M428L</td>
<td>3</td>
<td>0.629 ± 0.060</td>
<td>14</td>
</tr>
<tr>
<td>IgG1 T250Q/M428L</td>
<td>3</td>
<td>0.236 ± 0.013</td>
<td>37</td>
</tr>
</tbody>
</table>

Half-Life of Chimeric Antibodies in Mice

However, in this case we find no direct correlation of FcRn affinity with half-life.
One important question is what determines the \textit{in vivo} persistence of antibodies

An important role for FcRn has emerged

While it is clear that expression of FcRn is important for a long serum half-life for IgG and that altering the affinity of an Ab for FcRn can alter its half-life in some cases, it remains unclear what other factors contribute to the observed differences \textit{in vivo} persistence of different Abs
The FcγRs CD16, CD32 and CD64 play important roles in phagocytosis and ADCC. The inhibitory receptor, FcγRIIib plays a very important role in immune modulation. The affinity of an Ab for FcγRs can play an important role in its efficacy.
Complex of FcγRIII with Antibody (human IgG1)
IgG1 residues identified by site-directed as important for FcγR binding (alanine scanning)

Red: affected binding to all three receptors. The FcγRI site is comprised only of red residues

Magenta: improved binding to FcγRII and FcγRIIIA

Green: A. improved binding to FcγRII
       B. improved binding to FcγRIIA

Yellow: A. reduced bind to FcγRII
       B. reduced binding to FcγRIIIA

Although the FcγRs bind a similar region they are not identical

Shields et. al., JBC 276:6591-6604, 2001
Site-directed mutagenesis has been used to produce Abs with altered affinity for the different FcγRs.

In this example there has been an alteration in the relative binding to the activating receptor FcγRIII and the inhibitory receptor FcγRII.

### Table 1. FcγR affinity enhancements of Fc variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Alem AS [LOG(IC₅₀) (M)] fold</th>
<th>Tras AS [LOG(IC₅₀) (M)] fold</th>
<th>Tras SPR [Kᵦ] (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V158 Illa</td>
<td>F158 Illa</td>
<td>V158 Illa</td>
</tr>
<tr>
<td>WT</td>
<td>[−7.60 ± 0.02] 1</td>
<td>[−6.90 ± 0.06] 1</td>
<td>[−6.42 ± 0.06] 1</td>
</tr>
<tr>
<td>S298A/E333A/K334A†</td>
<td>[−8.71 ± 0.13] 13</td>
<td>[−8.01 ± 0.10] 13</td>
<td></td>
</tr>
<tr>
<td>S239D</td>
<td>[−8.72 ± 0.12] 13</td>
<td>[−7.72 ± 0.06] 7</td>
<td>[−7.65 ± 0.06] 17</td>
</tr>
<tr>
<td>I332F</td>
<td>[−8.61 ± 0.08] 10</td>
<td>[−7.89 ± 0.09] 10</td>
<td>[−7.22 ± 0.05] 6</td>
</tr>
<tr>
<td>S239D/I332E</td>
<td>[−9.44 ± 0.08] 70</td>
<td>[−8.70 ± 0.10] 63</td>
<td>[−8.83 ± 0.05] 254</td>
</tr>
<tr>
<td>S239D/I332E/A330L</td>
<td>[−9.66 ± 0.07] 115</td>
<td>[−9.12 ± 0.05] 169</td>
<td>[−8.99 ± 0.05] 370</td>
</tr>
</tbody>
</table>

*Table values provide LOG(IC₅₀) or Kᵦ [bracketed] values followed by folds relative to WT. Fold = IC₅₀variant/IC₅₀WT.
*Illa:IIb* = fold V158 FcγRIIlia/fold FcγRIIb for trastuzumab.
†Generated in a previous study (14) and used here for comparison.
The increased affinity for FcγRIII translates into more effective ADCC

Cell Based ADCC Against Cell Expressing Different Levels of Her2/neu

Gray: Wt Trastuzumab  
Blue S293D/I332E  
Tan S293D/I332E/A330L

*PNAS 2006;103;4005-4010*
Complement activation is also an important component of the antibody-mediated inflammatory response. Antibody-mediated complement activation contributes to many effects including cell lysis and opsonization.
Complement is activated when C1q binds to two adjacent Fcs of IgG. The C1q binding site is located in the CH2 domain.
C1q binding site of hIgG1

Mutations at K326 and E333 in CH2 alter C1q binding (filled) and CDC (open)

CDC of WIL2-5 lymphoma cells with human complement

Mutations in the C1q binding site can also be made to eliminate complement activation

*PNAS 2006;103;4005-4010*
Summary

Mutagenesis of the constant region of Abs can be used to produce Abs with altered functional properties including half-life, FcγR binding and complement activation.
All antibodies are glycoproteins and contain at least one N-linked carbohydrate.

Properties of antibodies are determined by both their amino acid sequence and their associated carbohydrate.
Glycosylation of IgG

2.8 N-linked oligosaccharides per IgG

2 are associated with the Fc region

The remainder are associated with the variable region
Carbohydrate Synthesis

Ribosome

Endoplasmic Reticulum

Cytoplasm

DOL

DOL

Carbohydrate Addition Site

Asn X Ser/Thr

X cannot be Pro

Glucose

Mannose

N-Acetyl Glucosamine

Asparagine

Galactose

Sialic Acid

Fucose

Endoplasmic Reticulum
As a consequence of this synthetic pathways, many different glycoforms are associated with antibodies.
The carbohydrate plays an important role in binding to FcγRs. IgG lacking carbohydrate does not bind.

The **structure** of the glycan can also influence the properties of the Ab.
Carbohydrates of different structure are added to the same Ab by different expressions systems.
The Structure of the Carbohydrate Influences FcγR Binding

![Graphs showing FcγR binding for KM3065 and Rituximab](image)

- KM3065
- Rituximab (CHO)
The structure of the carbohydrate influences ADCC
The presence of terminal sialic acid can also influence FcγR binding
The presence of carbohydrate in $C_H2$ is also required for complement activation.
Production Systems for Recombinant Antibodies

Mammalian Cell Lines: e.g. CHO and murine myelomas

Transgenic Animals
  Cattle
  Chickens (eggs)

Yeast

Bacteria (fragments)
Antibody like proteins can also be produced for many applications.

**Antibody Fusion Proteins**

- **H-Fusion**
- **C_{H1}-Fusion**
- **C_{H3}-Fusion**
- **NH_{2}-Fusion**

Drug delivery
Targeting molecules to sites such as tumors
Summary

It is possible to produce recombinant Abs with diverse properties

- Half-life
- ADCC
- Complement Activation

This can be approached by changing either the amino acid sequence or the structure of the attached carbohydrate.

Novel molecules such as Ab fusion proteins can also be made.

A challenge remains to identify the best Ab for the desired application.