Autoantibodies: Methods and Meaning

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Objectives

- Contrast the techniques of immunofluorescence and ELISA in detecting autoantibodies
- List the most common autoantibodies and their associated diseases
- Explain the importance of diluting samples when screening for autoantibodies
Are These Really Part of Clinical Chemistry?

• began as “immunology”, but much of “immunology” is now Clinical Chemistry:
  • IgG, IgA, IgM if not PEPs, IFEs
  • CRP, C3, C4

• methodology is rapidly becoming standard, as well as novel, immunoassays

• no people better qualified than clinical chemists to oversee these assays
Autoantibodies

• antibodies directed against “self”

• give rise to a number of different diseases
  – Some relatively common (rheumatoid arthritis)
  – Some relatively serious (systemic lupus erythematosis)

• help to understand, diagnose, and monitor disease
<table>
<thead>
<tr>
<th>Method Used</th>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirect Immunofluorescence</td>
<td>ANCA</td>
<td>Anti-Neutrophilic Cytoplasmic Antibody</td>
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<tr>
<td></td>
<td>ANA</td>
<td>Anti-Nuclear Antibody</td>
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<tr>
<td></td>
<td>AMA</td>
<td>Anti-Mitochondrial Antibody</td>
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<td>APCA</td>
<td>Anti-Parietal Cell Antibody</td>
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<td>&quot;ELISA&quot;</td>
<td>anti-TTG</td>
<td>Anti-Tissue Transglutaminase Antibody</td>
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<td></td>
<td>anti-DGP</td>
<td>Anti-Deamidated Gliadin Peptide</td>
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<td></td>
<td>anti-TPO</td>
<td>Anti-Thyroid Peroxidase Antibody</td>
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<td></td>
<td>anti-Tg</td>
<td>Anti-Thyroglobulin Antibody</td>
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<td></td>
<td>RF</td>
<td>Rheumatoid Factor</td>
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<tr>
<td></td>
<td>anti-CCP</td>
<td>Anti-Cyclic Citrullinated Antibody</td>
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Methods in Use

• Indirect Immunofluorescence

• Immunoassays
  – Standard ELISAs
  – “Multiplex” Immunoassays
Indirect Immunofluorescence
(The “Gold” Standard or At Least the Original)

- Multiple well slides; different cell types on different slides
- Each well contains thousands of antigens

Adapted from Bio-Rad Autoimmune TUTOR
Adapted from Bio-Rad AutoimmuneTUTOR

- Add patient serum
- Wash slide
- Add conjugate (labeled anti-IgG)
- Wash slide, then read

Thousands of antigens
Immunofluorescence Technique Notes

- Using different substrates (cell types), one “captures” antibodies with different specificities.
Substrate Specificity

Hep2 Cells: Anti-Nuclear Antibodies (ANA)

Mouse Stomach/Kidney Cells:
  Anti-Mitochondrial Antibodies (AMA)
  Anti-Smooth Muscle Antibodies (ASMA)
  Anti-Parietal Cell Antibodies (APCA)

Neutrophils
  Anti-Neutrophil Cytoplasmic Antibodies (ANCA)

Adapted from Bio-Rad AutoimmuneTUTOR
Substrate Specificity

anti-mitochondrial antibody

anti-smooth muscle antibody

parietal cells

smooth muscle

kidney

Adapted from Bio-Rad AutoimmuneTUTOR
Immunofluorescence Technique Notes

• Using different substrates (cell types), one “captures” antibodies with different specificities

• In some cases, the “pattern” of staining may be helpful, suggesting one disease over another
Patterns Can Sometimes Be Helpful (ANCA)

Cytoplasmic ANCA (C-ANCA) Pattern

- strongly suggests Wegener’s Granulomatosis

Perinuclear ANCA (P-ANCA) Pattern

- suggests vasculitis other than Wegener’s Granulomatosis

Adapted from Bio-Rad AutoimmuneTUTOR
Patterns Can Sometimes Be Helpful (ANA)
Other Notes on “Patterns”

• Although patterns are frequently definitive, sometimes they can be ambiguous, so the interpretations become subjective.

• In some cases, more than one autoantibody is present, which can definitely obscure the patterns.

• If more specificity is needed, it is probably better to rely on immunoassays directed at specific antigens (later).
Immunofluorescence Technique Notes

• Using different substrates (cell types), one “captures” antibodies with different specificities

• In some cases, the “pattern” of staining may be helpful, suggesting one disease over another

• Quantitation is crude (by clinical chemistry standards):
  – make serial dilutions until the result is negative
  – i.e., 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, etc.
Immunofluorescence Technique Notes

- Using different substrates (cell types), one “captures” antibodies with different specificities.

- In some cases, the “pattern” of staining may be helpful, suggesting one disease over another.

- Quantitation is crude (by clinical chemistry standards):
  - make serial dilutions until the result is negative.

- Autoantibodies occur frequently in healthy individuals:
  - what distinguishes disease from normal is “titer”.
Distribution of Values from Patients with Disease

cut-off that detects 99% of patients
Add Distribution of Values from Healthy People

1% false negative
20% false positive

7.5% false negative
2.5% false positive
One Specific Example: ANA

- At 1:40 dilution, 20% of normals are POSITIVE
- At 1:160 dilution, 5% of normals are POSITIVE

- In the absence of strong clinical suspicion of an autoantibody-mediated disease (i.e., for “screening”), it’s probably best to use 1:160 as your lowest titer

- Most labs, including mine, start at 1:40, because the physicians “don’t want to miss any cases”
- My compromise: all “positive” ANAs are reported with the disclaimer that roughly 20% of healthy people will have positive ANAs with titers of 1:40 or 1:80
Methods in Use

• Indirect Immunofluorescence

• Immunoassays
  – Standard ELISAs
  – “Multiplex” Immunoassays
ELISAs For Distinguishing P-ANCA and C-ANCA

- we saw the bottom halves of these images earlier
- using ELISAs for MPO and PR3 accomplish the same thing

Adapted from Bio-Rad AutoimmuneTUTOR
So Why Not Just Do the ELISAs?(1)

- Some samples have positive indirect immunofluorescence but are negative for the specific antigens
  - e.g., positive ANCA but negative for MPO and PR3
  - \(\Rightarrow\) defined as “atypical ANCA”
  - clearly, biologic substrate has many more antigens
  - the clinical significance of such antibodies is unclear
  - but these “atypical ANCAs” are associated with vasculitis

- This phenomenon occurs with many, if not all, autoantibodies; it is not limited to ANCA
So Why Not Just Do the ELISAs?(2)

• In most cases, it is relatively expensive to multiple ELISAs:
  – typically, the number of test requests is relatively small, so, most of the wells in your run will be standards and controls
  – for example, assume 3 patient samples for ANCA
    • you might have to include 6 standards and 2 controls
    • ➔ 8 of 11 samples for the ELISA would be “overhead”
    • and, you’d have to run 2 ELISAs (MPO, PR3)

• It may be less expensive to screen with IFA, and then confirm with ELISA
  – but ELISA becomes even less efficient if there’s only 1 sample
  – one hopes there is only 1 positive sample per week, though
  – in practice, overwhelming majority of samples are NEGATIVE
Immunoblot (Rather Than ELISA) for Confirmation?

- instead of running separate ELISAs, run immunoblots of PR3 and MPO alongside the original ANCA!
- ➔ immediate confirmation
- ➔ no (or very little) overhead

adapted from www.euroimmununus.com
Methods in Use

• Indirect Immunofluorescence

• Immunoassays
  – Standard ELISAs
  – “Multiplex” Immunoassays
“Multiplex” Immunoassays

• run many immunoassays simultaneously
  – for ANCA, run MPO + PR3
  – for ANA, run dsDNA, SSA, SSB, Sm, RNP, SCL-70

• instead of standard microtiter plate ELISAs, use novel technologies:
  – e.g., Luminex and euroimmun
  – if any assay is positive, call the overall test positive and give the specificity (e.g., ANA positive, dsDNA positive)
Multiplex Immunoassays Based on Luminex Technology

- beads of up to 100 different “colors”
- each different “color” is coated with a different antigen
- patient samples are incubated in a single well, with a mixture of all relevant beads for the assay in question
- if any antibodies are present in the sample, they will bind to the corresponding bead (e.g., anti dsDNA binds to yellow beads)
- a fluorescence labeled anti-IgG is added, which binds to any relevant patient antibodies, forming a “sandwich”: (bead with antigen)-(patient antibody)-(fluorescent anti-IgG)
- an aliquot of the reaction mixture is run through a flow cytometer, where the “color” of each bead is assessed, along with the presence of any IgG
- in this way, the flow cytometer know which beads (antigens) had antibody bound to them
Multiplex Immunoassay Based on EuroLine Technology (not FDA-approved)

- multiple antigens
- blotted onto a single strip
- separated in space
- incubate with patient sample
- wash
- incubate with enzyme-labeled anti-IgG
- wash
- add substrate
- read
Potential Downsides of Multiplex Format

• May be expensive:
  – Multiple standard ELISAs seem expensive
  – Novel format may be even more expensive, except that the labor costs are minimal
  – Assumes that every ELISA should be run on every sample

• May miss “atypical” positives
  – Samples positive by indirect immunofluorescence but negative by ELISA
  – They do exist, but their significance is debated
Celiac Disease

- relatively common (may be as high as 1 in 133 Caucasians)
- when susceptible patients eat gluten, (a protein found in wheat, rye, and barley), they make autoantibodies that attack the villi of the small intestine
- this results in malabsorption diarrhea, gas, bloating inability to absorb nutrients, which can lead to vitamin deficiencies, weight loss, etc.
- also associated with increased risk of several cancers
Celiac Disease Testing

- several different tests have been used:
  - Anti-Gliadin Antibodies (ELISA)
  - Anti-Endomysial Antibodies (indirect immunofluorescence)
  - Anti-Tissue Transglutaminase (Anti-TTG) (ELISA)
  - Anti-Deamidated Gliadin Peptide (Anti-DGP) (ELISA)

- currently, the test of choice is IgA anti-TTG:
  - that is, only IgA antibodies directed against TTG
  - IgG anti-TTG antibodies appear to be less specific
Caveats: Celiac Disease Testing

- false negative IgA anti-TTG results:
  - patients with IgA deficiency (1 in 400)
    ➞ check IgA levels in patients with negative IgA anti-TTG
    for IgA deficient patients, retest with an alternative
    celiac autoantibody test
    e.g., anti-DGP, which includes IgG and IgA antibodies

- false negative results (all tests) in genuine celiac patients
  - patients on gluten-free diets
    ➞ if a patient has implemented a gluten-free diet on his own,
      his autoantibodies may well disappear
Thyroid Antibodies

• Many different tests are classified as “thyroid antibodies”

• The two you should definitely know about are:
  – Anti-Thyroid Peroxidase (anti-TPO)
  – Anti-Thyroglobulin (anti-Tg)

• Both are done by ELISA (or automated variants thereof)
Thyroid Antibodies: Anti-TPO

- Anti-TPO is the test of choice for autoimmune thyroiditis
  - Grave’s Disease
    patients usually present with hyperthyroidism
    sometimes, they can extremely ill
    undetectable TSH, very high Free T4
  - Hashimoto’s Disease
    autoantibodies destroy thyroid tissue
    ultimately leads to hypothyroidism
    high TSH, low Free T4
Thyroid Antibodies: Anti-Tg

- Anti-Tg should only be run to ensure accurate Tg

- Following thyroid gland removal for certain cancers (well differentiated papillary carcinoma), Tg serves as a tumor marker for recurrence

- Since the Tg assay uses anti-Tg in the reagent system, (e.g., capture antibody), the presence of anti-Tg in the patient’s serum will confound the assay

- In the presence of anti-Tg, it is difficult, if not impossible, to interpret the results of a Tg assay (and such results should probably not be reported)
Rheumatoid Factor

- IgM autoantibody directed against IgG
- associated with Rheumatoid Arthritis, but not causally related to the disease
- measured by immunoturbidimetry or nephelometry rather than by indirect immunofluorescence or ELISA
- like most autoantibodies, RF lacks sensitivity and specificity
- Anti-Cyclic Citrullinated Peptide Antibody (anti-CCP)
  - a newer assay
  - said to be more sensitive and more specific than RF
  - measured by ELISA (and automated variants)
## Disease Associations

<table>
<thead>
<tr>
<th>Indirect Immuno-fluorescence</th>
<th>Antigen (ELISA target)</th>
<th>Associated Disease(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANCA</td>
<td>C-ANCA</td>
<td>Wegener's</td>
</tr>
<tr>
<td></td>
<td>P-ANCA</td>
<td>Other Vasculitis, Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>ANA</td>
<td>dsDNA</td>
<td>Systemic Lupus Erythematosis</td>
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<td></td>
<td>Ro/SSA</td>
<td>Systemic Lupus Erythematosis</td>
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<td></td>
<td>La/SSB</td>
<td>Sjogren’s Syndrome</td>
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<tr>
<td></td>
<td>Sm</td>
<td>Systemic Lupus Erythematosi</td>
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<td></td>
<td>Jo1</td>
<td>Polymyositis</td>
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<td></td>
<td>SCL-70</td>
<td>Scleroderma</td>
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<tr>
<td>AMA</td>
<td></td>
<td>Primary Biliary Cirrhosis</td>
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<tr>
<td>ASMA</td>
<td></td>
<td>Autoimmune Hepatitis</td>
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<tr>
<td>APCA</td>
<td></td>
<td>Pernicious Anemia</td>
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<tr>
<td></td>
<td>TTG</td>
<td>Celiac Disease</td>
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<tr>
<td></td>
<td>TPO</td>
<td>Thyroiditis (Graves and Hashimoto's)</td>
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<tr>
<td></td>
<td>Tg</td>
<td>QA for use with Tg (recurrence of thyroid) cancer</td>
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<tr>
<td></td>
<td>RF</td>
<td>Rheumatoid Arthritis</td>
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Which of the following combinations of autoantibody and disease is incorrect?

A) ANA: Systemic Lupus Erythematosi
B) ANCA: Wegner’s Granulomatosis
C) ANA: Primary Biliary Cirrhosis
D) anti-TTG: Celiac Disease
Self-Assessment Question 2

Which of the following methods is **not** typically used for autoantibody measurement?

A) Indirect Immunofluorescence  
B) ELISA  
C) Multiplex ELISA  
D) Mass Spectrometry
Self-Assessment Question 3

All of the following are “weird but true” except:

A) You shouldn’t measure Tg unless you’ve proved that anti-Tg is not present
B) Rheumatoid Factor is an autoantibody directed against normal IgM molecules
C) IgA deficiency complicates screening for celiac disease
D) A large minority of healthy people positive ANAs
1. (C) ANA: Primary Biliary Cirrhosis

2. (D) Mass Spectrometry

3. (B) Rheumatoid Factor is an autoantibody directed against normal IgM molecules