Autoimmune Disease:
So Many Tests, But Not So Complicated

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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 erythematosus)
- help to understand, diagnose, and monitor disease

Autoantibodies We Will Cover

<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>
</tr>
<tr>
<td></td>
<td>AMA</td>
<td>Anti-Mitochondrial Antibody</td>
</tr>
<tr>
<td></td>
<td>ASMA</td>
<td>Anti-Smooth Muscle Antibody</td>
</tr>
<tr>
<td></td>
<td>APCA</td>
<td>Anti-Parietal Cell Antibody</td>
</tr>
<tr>
<td>&quot;ELISA&quot;</td>
<td>anti-TG</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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<tr>
<td></td>
<td>anti-TPO</td>
<td>Anti-Thyroid Peroxidase Antibody</td>
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<tr>
<td></td>
<td>anti-Tg</td>
<td>Anti-Thyroglobulin Antibody</td>
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<tr>
<td></td>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td></td>
<td>anti-CCP</td>
<td>Anti-Cyclic Citrullinated Antibody</td>
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Case History

- A 60 year old woman with chronic knee pain is referred to a rheumatologist, who orders several autoantibody tests
- The patient sees her results online, noticing that one test is “positive”
  ANA: POSITIVE, Speckled Pattern, Titer 1:40
- After reading more about the test online, she infers that she must have SLE (Systemic Lupus Erythematosus) and envisions a very poor prognosis
- She is not scheduled to see the rheumatologist in follow-up for two weeks
Methods in Use

- Indirect Immunofluorescence
  - Immunoassays
    - Standard ELISAs
    - "Multiplex" Immunoassays

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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

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Indirect Immunofluorescence
(The “Gold” Standard or At Least the Original)

- add patient serum
- wash slide
- add conjugate (labeled anti-IgG)
- wash slide, then read

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Adapted from Bio-Rad AutoimmuneTUTOR
**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)

**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
• 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.

• Autoantibodies occur frequently in healthy individuals
  – what distinguishes disease from normal is "titer"
Add Distribution of Values from Healthy People

![Graph showing distribution of values with 1% false negative, 20% false positive, 7.5% false negative, and 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

Back to Our Patient with Knee Pain

- ANA was “positive” at 1:40 titer
- History was not suggestive of SLE (or any other “collagen vascular disease”)
- Most likely, a false positive
- Predictive Value of a Positive Test (more about this on Wednesday evening)
  - Given Prevalence 2%, Sensitivity 99%, Specificity 80%
  - $PV(+) = 8\%$
  - in other words, 92% of positives are false positives
**Methods in Use**

- Indirect Immunofluorescence
- Immunoassays
  - Standard ELISAs
  - "Multiplex" Immunoassays

**ELISA Technique**

**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
  - 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

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

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 Immunoassay</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>Ro/SSA</td>
<td>Systemic Lupus Erythematosus</td>
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<tr>
<td></td>
<td>La/SSB</td>
<td>Sjogren’s Syndrome</td>
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<tr>
<td></td>
<td>Sm</td>
<td>Systemic Lupus Erythematosis</td>
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<tr>
<td></td>
<td>Jo1</td>
<td>Polymyositis</td>
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<tr>
<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>APMA</td>
<td></td>
<td>Autoimmune Hepatitis</td>
</tr>
<tr>
<td>ACPA</td>
<td></td>
<td>Perinuclear ANA</td>
</tr>
<tr>
<td>TgA</td>
<td></td>
<td>Coeliac Disease</td>
</tr>
<tr>
<td>TPO</td>
<td></td>
<td>Thyrotrypin (Hashimoto’s)</td>
</tr>
<tr>
<td>Tg</td>
<td></td>
<td>Ok for use with Tg (occurrence of thyroid cancer)</td>
</tr>
<tr>
<td>RF</td>
<td></td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>CCP</td>
<td></td>
<td>Rheumatoid Arthritis</td>
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Self-Assessment Question 1
Which of the following combinations of autoantibody and disease is incorrect?
A) ANA: Systemic Lupus Erythematosus
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