Advances in Laboratory Testing for Rheumatic Diseases 2010

Jonathan Graf, M.D.
Assistant Clinical Professor of Medicine
University of California, San Francisco
Division of Rheumatology
San Francisco General Hospital

The black hole of medical knowledge: An internist’s view of rheumatologic lab tests

The typical ABIM Board Examination Question on Rheumatology Lab Testing:

No Idea

Rh. Factor

ANA

ANCA

Updates in Testing for Rheumatic Diseases

Chapter 1: Rheumatoid Arthritis: Recent advances in diagnostic and prognostic testing

Chapter 2: Systemic Lupus Erythematosus: Two steps forward and perhaps a step backwards
Rheumatoid Arthritis

- Chronic, inflammatory, predominantly small joint arthritis
- Affects up to 1% of the US population
- Female:Male predominance of 3:1
- Disability costs are high, both in terms of direct and indirect medical costs
  - 35% of patients with 10 years disease duration are work-disabled
  - Decline from 50% rate reported in 1987

RA: Chronic Joint Destruction and Disability – What We Try to Prevent

Improving Outcomes in RA: Three Pillars

- Improvement in timely and accurate diagnosis
- Development of better tools to predict disease severity
- Improvement in therapy
Early RA: The Window of Opportunity to Intervene

The Window of Opportunity Eventually Closes for Many….

- Chronic disease progression leads to permanent joint deformity, destruction, and disability
- Empirically, RA is a different disease the longer disease activity progresses without effective control
  - More difficult to suppress activity and treat
  - More extra-articular disease?

ACR Criteria for the Classification of Rheumatoid Arthritis
(≥4 criteria required; 1-4 must be present > 6 wks)
- Morning stiffness > 1 hr
- Arthritis of 3 or more joint areas
- Arthritis of wrists, MCPs, and/or PIPs
- Symmetric arthritis
- Rheumatoid nodules
- Serum rheumatoid factor
- Radiographic changes

Limitations of ACR Classification Criteria for the diagnosis of early RA
- Developed for the classification of patients with longstanding disease (for clinical studies, not diagnosis)
- For early RA:
  - Specificity: 90%
  - Limited sensitivity: 40-65%
- Relying on criteria to make a diagnosis of RA can lead to delayed or inappropriate diagnosis
Diagnosis of early RA by ACR criteria
van Gaalen et al Arth Rheum 50: 709, 2004

936 patients with early inflammatory arthritis

Initial evaluation After 3 years

205 RA by ACR criteria
318 “undifferentiated arthritis”
127 RA
413 other diagnoses

Factors predictive of progression from undifferentiated arthritis to RA
van Gaalen et al Arth Rheum 50: 709, 2004

At initial evaluation OR (95% CI)
Positive rheumatoid factor 1.7 (0.5-5.6)
Positive anti-CCP antibody 38.6 (9.9-151.0)

Posttranslational modification of proteins:
PADI converts arginine to citrulline

RA-associated autoantibodies that recognize peptides containing citrulline

Peptide sequence Antibody recognition
ESSRDGSRHPRSHD No
ESSRDGS_{cit}HPRSHD Yes

Actual citrullinated antigen is not known
Antibodies to citrullinated peptides in RA

- Detected by ELISAs using synthetic cyclic citrullinated peptides (CCP)
- Sensitivity for very early RA: 50%
- Sensitivity for early-later RA: 70-80%
- Specificity for RA: 95-98%

RF and anti-CCP testing in a cohort of 182 early RA patients
Quinn et al Rheumatology (Oxford) 45:478, 2006

Preclinical autoimmunity in RA: appearance of anti-CCP abs and RF prior to onset of arthritis
Nielen et al Arth Rheum 50: 380, 2004

Improving Outcomes in RA: Three Pillars

- Improvement in timely and accurate diagnosis
- Development of better tools to predict disease severity
  - Possible to predict which patients require more aggressive therapy up front?
- Improvement in therapy
Classic Predictors of Disease Severity

- More difficult to treat, destructive, extra-articular disease:
  - Rheumatoid factor positive
  - Erosive disease
  - Genetic factors: HLA DR4 with RR of 6:1
    - Turns out that some DR4 alleles do not confer risk
    - And some non-DR4 alleles do!
    - There is something that most DR risk alleles share in common!

HLA DR alleles: Shared Epitope Hypothesis for RA

<table>
<thead>
<tr>
<th>DRB1 allele</th>
<th>70</th>
<th>71</th>
<th>72</th>
<th>73</th>
<th>74</th>
</tr>
</thead>
<tbody>
<tr>
<td>0101</td>
<td>Q</td>
<td>R</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>0401</td>
<td>Q</td>
<td>K</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>0404</td>
<td>Q</td>
<td>R</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>0405</td>
<td>Q</td>
<td>R</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>0408</td>
<td>Q</td>
<td>R</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1402</td>
<td>Q</td>
<td>R</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1001</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>CONSENSUS</td>
<td>Q/R</td>
<td>R/K</td>
<td>R</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

The shared epitope (DRB1*0401)

HLA DR and rheumatoid arthritis: 25 years without clinical utility

- It’s actually this shared epitope that confers the risk of developing RA
- Homozygotes for SE (can mix and match) have more severe disease than do heterozygotes or those without DR4
- Still not practical to genotype all patients
Relationship between shared epitope and CCP antibodies in RA


Table 5. Association of HLA-DRB1 shared epitope (SE) alleles and anti-CCP autoantibodies in rheumatoid arthritis

<table>
<thead>
<tr>
<th>SE status</th>
<th>No. of carriers</th>
<th>Anti-CCP+</th>
<th>Anti-CCP-</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE+</td>
<td>41</td>
<td>34</td>
<td>6</td>
<td>13.3 (4.0-46.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SE-</td>
<td>37</td>
<td>36</td>
<td>1</td>
<td>4.7 (1.1-18.9)</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Suggests that homozygotes for SE get CCP+ RA, those without SE get CCP- RA

Presence of CCP antibodies, not SE, confers risk for more aggressive RA


Progression of joint damage in subgroups of early RA

Huizinga, Criswell et al. Arthritis Research & Therapy 7: 949, 2005

Is anti-CCP+ RA a different genetic disease than anti-CCP neg RA??


At least one copy of SE

- 42%
- 80%
- 49%

Presence of CCP antibodies, not SE, confers risk for more aggressive RA


Progression of joint damage in subgroups of early RA

Huizinga, Criswell et al. Arthritis Research & Therapy 7: 949, 2005

radiographic joint damage score

anti-CCP+ 

anti-CCP-
Anti-CCP status

- Anti-CCP positive RA patients are unique compared to anti-CCP negative patients
  - Shared epitope positive compared to controls
    - No additional contribution to risk of developing RA from SE independent of CCP status (data not shown)
  - More erosive disease
  - More progressive course of disease (radiographically)

Is rheumatoid arthritis a single disease?

<table>
<thead>
<tr>
<th></th>
<th>RA #1</th>
<th>RA #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CCP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Erosive dz</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Summary: Clinical utility of the anti-CCP antibody test

- Diagnosis:
  - Clinical suspicion of rheumatoid arthritis
  - Early, undifferentiated inflammatory arthritis
  - Distinguish RA from other RF+ polyarthritis
- Not useful to monitor disease activity
- Best single predictor for destructive disease in patients with early onset RA
Sorting out the ANA Puzzle

So that ANAs make sense

Advances in Laboratory Diagnoses of Rheumatic Diseases

• Anti Citrullinated Peptide Antibodies in the diagnosis, prognosis, and management of rheumatoid arthritis

• Automated autoantibody screening

Automation: Coming to a lab near you

• Allows for high throughput testing
  – Many patient samples
  – Many tests per sample
• Cheaper
• Less prone to human “interpretation”; standardized
• Actually been around for a while
  – Automated Chem panels
  – CBC’s
• Increasing use of lab automation for more “complex tests”
  – Serologies
  – Anti-nuclear antibodies
What’s in Ourselves??
What’s in a Cell?

- Cell membrane
  - Phospholipids

- Cytoplasm
  - Mitochondria

- Nucleus
  - Nucleic Acids
  - Histones

What is an Anti-Nuclear Antibody?

- Autoantibodies directed specifically against intra-nuclear antigens
- Most commonly (not always) detected by the technique of immunofluorescence on whole cells
- Specific antigen may or may not be known (most ANA’s aren’t known – only seen by staining)
- Smaller handful of specific antibodies target known nuclear antigens associated with rheumatic and other diseases

What is Indirect Immunofluorescence??

- Hep-2 cells fixed to slide & permeabilized
- Incubated in patient serum
- Washed vigorously to remove serum
- Fluorescently labeled Anti-hum Ig secondary Ab
- Wash again
- Detect florescence of bound secondary Ab

ANA Patterns

Depends upon what molecule(s) are recognized by patient antibodies
- DNA is homogeneously distributed
- Centromere’s seen in dividing cells
- Extractable nuclear antigens are speckled throughout cell
What does an indirect immunofluorescence look like?

ANA Patterns: Speckled

ANA Patterns: Nucleolar

ANAs by immunofluorescence

- Costly
- Low throughput
  - One test on one sample per patient
  - Time consuming
- Labor intensive
  - Someone has to prepare the test, titer out serum samples, etc...
  - Someone has to read the slide and make a judgment
  - Subject to human interpretation (what’s positive/what’s background?)
- Hep-2 cell lines contain antigens of unknown significance that lower specificity of test
- When ANA IIF is positive, actual autoantibody target is not known
  - Pattern may be suggestive, but not diagnostic
  - Is it something important like anti-Smith or is it a non-specific antigen?
The ANA IIF Test Characteristics
Sensitive for SLE, Not Specific

- Modern Hep-2 ANA IIF is nearly 99% sensitive for SLE (90% sensitive for scleroderma, etc.)
- ANA IIF is not nearly as specific for SLE as it is sensitive
  - Autoimmune thyroid disease
  - Other Collagen-Vascular diseases (>90% of SSc)
  - Medications
  - Malignancies
  - Infections (viral)
  - Normal people (especially low titers)
Multiplex Testing: Technique

- Very tiny color-coded beads are coated with a specific antigen, such that the bead’s color and antigen are linked together
- Several thousand of each specific antigen-coated bead are combined into a single reagent, which can be used for detecting multiple antibodies from one patient sample
- A second color-labeled Anti-human IgG is used to detect autoantibodies that bind to the beads
- The beads flow through a laser detector that detects 2 colors: that of the specific bead and that of any bound antibodies

A Crude Look at Automated ANAs

Automated MultiPlex ANA Assays

- Can tell you with good specificity if ANA+
  - Laser beam and detector vs. human eye
- Can tell the specific antigen target of the anti-nuclear antibody(ies)
- Can tell you multiple antibody specificities with one sample (based on numbers of different antigen coated beads)
- Can run multiple different patient samples rapidly
Automated ANA Testing

Hmmm: If the test is more specific, can tell you specifically what the ANA is (e.g., Anti-dsDNA), is automated, and only gives you the autoantibodies that are most clinically relevant, then what’s the problem?

Pitfalls of Automated ANA’s

• Well, there’s no mention of sensitivity!

• What about the 100’s of other antigens in a nucleus that stain on immunofluorescence?
  – Although less specific, antibodies against any of these unknown antigens may be relevant when using the ANA to diagnose a collagen vascular disease like SLE (sensitivity is 99%)

• What about the native shapes of antigens inside living cells vs. synthetic antigens in a machine?
  – Antibodies that recognize an antigen in a cell may not recognize an antigen squished onto a synthetic bead

An example: dsDNA may look different inside a cell than on a bead: Does this matter??

Chromatin inside a Hep-2 Nucleus: Complex shapes recognized by anti-dsDNA antibodies

dsDNA linked to a bead??

Representative Case

• 47 year old female who presented with a progressive sensory neuropathy, cytopenias, and an ischemic rash associated with livedo reticularis

  • Suspicion for SLE but autoantibody testing was repeatedly negative
Clinical Case from Massachusetts General Hospital

<table>
<thead>
<tr>
<th>Euphemala andromedae (serum)</th>
<th>0-10</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-nuclear antibodies (ANA)</td>
<td>Qualitative</td>
<td>&lt;10 dilution, negative</td>
<td>&lt;10 dilution, negative</td>
</tr>
<tr>
<td>Speckled pattern</td>
<td>Negative</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Anti-DNA antibody</td>
<td>&lt;10 dilution, negative</td>
<td>&lt;10 dilution, negative</td>
<td></td>
</tr>
<tr>
<td>Anti-Smith antibody</td>
<td>&lt;10 dilution, negative</td>
<td>&lt;10 dilution, negative</td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA antibody</td>
<td>&lt;10 dilution, negative</td>
<td>&lt;10 dilution, negative</td>
<td></td>
</tr>
</tbody>
</table>

Key points:
1. ANA's detected by repeat with IIF
   - HIGH TITER
   - Likely not new seroconversion
   - Likely speckled antigen not represented in automated ANA

2. Anti-dsDNA (which had been negative in the automated assay) was detected using a different method (antigen preparation matters!!!)

NEJM Case 2009

Dr. Donald B. Bloch: On repeat testing at this hospital, ANAs were detected by indirect immunofluorescence assay (titer, 1:1280), in a speckled pattern. Subsequently, antibody to double-stranded DNA (dsDNA) was detected (titer, 1:40), enzyme immunoassays for antibodies to Ro (SS-A), La (SS-B), Smith, and U1-RNP were negative. A second serum sample from this patient was retested in our laboratory, and the positive ANA result was confirmed.

Key points:
1. ANA's detected by repeat with IIF
   - HIGH TITER
   - Likely not new seroconversion
   - Likely speckled antigen not represented in automated ANA

2. Anti-dsDNA (which had been negative in the automated assay) was detected using a different method (antigen preparation matters!!!)

Automated ANAs lack Sensitivity

Automated ANA/ENA Screening (EliA) as an alternative to IIF (HEp-2) in daily routine

Baptista-Fernandes, A.M., Matos-Ferreira, E., Correia-Junior, L. and Martins, A.P.

1. Laboratory Immunology, Hospital de Santa Cruz, Lisbon, Portugal
2. Laboratory Immunology, Hospital de Santa Cruz, Lisbon, Portugal
3. Laboratory Immunology, Hospital de Santa Cruz, Lisbon, Portugal

Table 1: Sensitivity, specificity, and likelihood ratios for the detection of ANAs with EliA vs. IIF (HEp-2) in daily routine

<table>
<thead>
<tr>
<th>ANA</th>
<th>EliA</th>
<th>IIF (HEp-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>Specificity (%)</td>
<td>Positive Likelihood Ratio</td>
</tr>
<tr>
<td>ANA</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>ENA</td>
<td>75</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 1: Sensitivity, specificity, and likelihood ratios for the detection of ANAs with EliA vs. IIF (HEp-2) in daily routine.

EliA Journal

The 4th International Conference on Immunology
"The main problem (among many others) was that multiple autoantibodies can be present in the sera of SLE patients that are not necessarily detected by the antigens that coat the beads. So many patients with documented SLE tested as ANA negative, and the diagnosis of SLE was missed among so many hospital admissions, that all 32 rheumatologists in our division signed a petition asking the Department of Clinical Pathology to reconsider the use of this new test."

**ACR: Position Statement 2/2009**

- The ANA by immunofluorescence is the gold standard
- Newer automated ANA’s that test for handfuls of antigens may miss 100’s of potential autoantigens screened by IIF
- Laboratories should indicate which method they use

**What should you do??**

- Be aware of the method your lab uses
  - This can be very tricky as different insurance companies use specific labs
  - Most of the major reference labs use automated ANA testing now unless you ask!
- Know the patient whom you’re testing
  - What is the pre-test likelihood they have a rheumatic disease like SLE??
  - If there remains a high pre-test likelihood of disease, and automated ANA testing is negative, then re-test the patient with an IIF ANA