ANTI-dsDNA: A SHORT HISTORY AND UPDATE

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Disclosure

Dr. Marvin Fritzler is or has been a consultant to Inova Diagnostics Inc., Werfen BioRad, Euroimmun GmbH, Mikrogen GmbH, Dr. Fooke Laboratorien GmbH, ImmunoConcepts, GSK Canada, Amgen, Roche and Pfizer. He is the Director of Mitogen Advanced Diagnostics Laboratory.
OUTLINE

• Discovery of anti-DNA antibodies.
• The origin of anti-DNA
• The spectrum of anti-DNA antibodies
• Clinical applications
• Immunoassays to detect anti-dsDNA
• Future considerations
What is anti-DNA?

Bev Doolittle “Red Fox”
Good News & Bad News: The Kodachrome Legacy

First the bad news

- Paul Simon:
  “If I look back on all the crap I learned in high school, it’s a wonder I can think at all.”

Now the good news:

“Kodachrome, gives those nice bright colors, They give us the greens of summers, Makes you think all the world's a sunny day, oh yah!”

“Everything looks worse in black and white”
A Short History of Anti-DNA

• 1957: Holman & Kunkel show that DNase of deoxyribonucleoprotein (DNP) eliminated the LE cell.
  *Science 126: 162-3, 1957*

• 1966: Tan, Schur, Carr et al. DNA and anti-DNA in SLE
  *J Clin Invest 45: 1732-40, 1966*

• 1967: Koffler, Schur & Kunkel elute anti-DNA from SLE kidney.
  *J Exp Med 126: 608-24, 1967*

• 1982: anti-dsDNA included in ARA Revised SLE Criteria
  *Arthritis Rheum 25: 1271-7, 1982*

• 2012: anti-dsDNA in SLICC criteria (SLICC-12)
  *Arthritis Rheum 64: 2677, 2012*
<table>
<thead>
<tr>
<th>Immunologic Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>10. Anti-DNA: antibody to native DNA in abnormal titer</td>
</tr>
<tr>
<td>2. Anti-Sm: presence of antibody to Sm nuclear antigen</td>
</tr>
<tr>
<td>3. Positive finding of antiphospholipid antibodies on:</td>
</tr>
<tr>
<td>1. an abnormal serum level of IgG or IgM anticardiolipin antibodies,</td>
</tr>
<tr>
<td>2. a positive test result for lupus anticoagulant using a standard method, or</td>
</tr>
<tr>
<td>3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test</td>
</tr>
</tbody>
</table>

No detection method mentioned
SLICC Criteria 2012

**Immunological Criteria**

1. ANA above laboratory reference range

2. Anti-dsDNA above laboratory reference range, except ELISA: twice above laboratory reference range

3. Anti-Sm

4. Antiphospholipid antibody: any of the following
   - lupus anticoagulant
   - false-positive RPR
   - medium or high titer anticardiolipin (IgA, IgG or IgM)
   - anti-\(\beta_2\) glycoprotein I (IgA, IgG or IgM)

5. Low complement
   - low C3
   - low C4
   - low CH50

6. Direct Coombs test *in the absence of hemolytic anemia*
The Origin of anti-DNA

• Two phases:
  1. **Antigenicity/breaking tolerance** vs. **driving** the B cell anti-DNA
     Which DNA is the original ‘stimulus’ for anti-DNA response via TLR9?
  2. **Driver** of B cell response: ‘**Native** DNA” (nuclear, mitochondrial)
  3. Although…Molecular ‘mimics’ of DNA: Phospholipids, proteins (polyoma large T antigen, HCMVpp65428-437, *T. cruzii* Fus 1, entactin, laminin, α-actinin)

• **Antigenicity** dependent on:
  • **Protein binding**
  • **Sequence**
  • Base methylation: Important in epigenetics
  • Backbone structure
  • Strandedness
  • Intracellular location
  • Extracellular location: cell death, microbodies, mitochondrial release

The “Drivers” of anti-DNA Responses

D. S. Pisetsky. Anti-DNA antibodies - quintessential biomarkers of SLE.


Note: Oxidized mitochondrial DNA released from stimulated PMN>>DAMP

The spectrum of nucleic acid antibodies

- Bases (purines, pyrimidines)
  - ssDNA, ssRNA
- Sugar-Phosphate backbone
  - ssDNA, dsDNA, RNA
- Double helix
  - dsDNA
- dsRNA
- DNA–protein complex (nucleosome)
- Other DNA conformations:
  - Z (left handed), cruciform, “kinked”, DNA/RNA hybrids, triplex, elongated

dsDNA for Immunoassays

• Recombinant dsDNA
  • Circular bacterial plasmids, no ssDNA or proteins

• Synthetic dsDNA
  • Design excludes the “primary” presence of ssDNA or proteins
  • Antigen used in the QUANTA Flash chemiluminescence assay

• “Native” dsDNA
  • For solid phase assays: Calf thymus, salmon sperm – need to ensure the purification process doesn’t leave ssDNA or histone contamination
  • For IIF assays (CLIFT) – *Crithidia luciliae* kinetoplast is an intracellular organelle lacking histones and no ssDNA….but what else is there?
Anti-dsDNA Antigen for Immunoassays

Take home message regarding the antigen source is:

• Source of the dsDNA is not critical as long as there is no ssDNA, histone, phospholipid or other contamination.

• DNA sequence is not a factor...maybe?

• BUT pure dsDNA is unlikely to occur in nature, hence:
  • “Testing for anti-dsDNA antibodies using pure dsDNA as target antigen is, by definition, an artificial analytical approach.” (Rekvig O, Clin Exp Immunol 179:75, 2014)
Anti-dsDNA Immunoassays

- Of Historical Interest
  - Hemagglutination:
    - DNase treated DNP bound to red cells
  - Immunodiffusion
  - Immunofluorescence
    - Peripheral/rim pattern: debunked
    - Metaphase chromosomes
      - histones and HMG proteins (acid extracted)
  - Fluorometry: ethidium bromide competitive assay
  - RIA
    - Millipore filter assay
    - Farr variant: Polyethlene glycol (PEG) IP assay
Contemporary Anti-dsDNA Immunoassays

- Radioimmunoassay (RIA)
  - Farr assay (ammonium sulfate precipitation)
- ELISA (note: typically dependent on poly L-lysine)
- Dot Blot/Line assays
- Bead-based assays
  - DNA beads or other solid phase assay (FEIA)
  - Addressable Laser Bead Immunoassays (ALBIA): Luminex platform
  - Chemiluminescence immunoassays (CIA): BioFLash platform
- IIF assays
  - Crithidia lucilliae (CLIFT)
CLIFT: Comments

• SLE: High specificity (>90%); Low sensitivity <30%

• ~1/200 +ve Kinetoplast staining — ANA negative

• Unique epitope of ‘kinked’ DNA

• Kinetoplast is a modified mitochondrion
  • Concatenated maxi- and mini-micro-circles
    • Maxi encode Oxid Phosphor genes
    • Mini encode “guide” RNA: editosomes

• 60% PBC/AIH Overlap syndrome*
  Muratori, A. et al.

Nguyen Swain, Norman, Fritzler replicated findings BUT not anti-dsDNA +ve in other immunoassay (Abstract submitted)
Considerations Anti-dsDNA Assays

- **Source**: human/mammalian vs. bacterial vs. mitochondrial
- **Purity**
  - “Contaminating” ssDNA:
    - Anti-ssDNA can affinity mature to anti-dsDNA
    - DNase treated, closed circular DNA, synthetic
- **Secondary binding of cationic serum/plasma molecules??**
- Anti-dsDNA = monogamous bivalent binding
  (both Fab contact the same polynucleotide chain)
- Want to detect high avidity antibodies
  - correlated with diagnosis and higher probability of renal involvement in **SLE**
- **ANA negative but** anti-dsDNA positive sera
Anti-dsDNA as a biomarker

- Antecedent: Risk of disease
- Clinical: Case finding
- Diagnostic: “Intent to treat”
- Staging: Disease severity (SLEDAI)
- Prognostic: Disease course
- Predictive: Response to therapy
- IFN signature: Assay dependent?
Clinical Applications Anti-dsDNA

- “Marker” antibody in Systemic Lupus Erythematosus (SLE)
  - For most immunoassays specificity >80%
- One criterion for classification of SLE
  - ACR – 11 criteria; must have 4 to be classified as having SLE
  - SLICC – 17 criteria; must have 4 and at least one clinical and one immunological
- Linked to pathogenesis of SLE*
- Anti-dsDNA/DNA immune complexes activate complement
  - Deposited/form ed *in situ* in the glomerulus leading to inflammation and lupus nephritis
- Antibody levels fluctuate with disease activity
  - Associated with decreased C3 and C4: complement and anti-dsDNA levels used to indicate/predict SLE flare or relapse
  - Depends on the assay being used
- BUT.. transient anti-dsDNA can occur in the context of an infection. A single positive test in time might not be “diagnostic”.

Antecedent Factors: UCTD Evolving to SLE

CLINICAL
- Fever
- Discoid lupus
- Serositis
- Photosensitivity
- Leukopenia

SEROLOGY
- Homogeneous ANA
- Anti-dsDNA
- Anti-Sm
- Anti-cardiolipin
- Multiple SLE-related autoantibodies
# Autoantibody Profile of UCTD Evolving to SLE

<table>
<thead>
<tr>
<th>AUTOANTIBODY</th>
<th>RANGE %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>60 - 100</td>
</tr>
<tr>
<td>dsDNA</td>
<td>5 - 20</td>
</tr>
<tr>
<td>SSA/Ro60</td>
<td>10 – 30</td>
</tr>
<tr>
<td>SSB/La</td>
<td>0 – 5</td>
</tr>
<tr>
<td>Sm</td>
<td>0 – 5</td>
</tr>
<tr>
<td>U1RNP</td>
<td>0 - 30</td>
</tr>
<tr>
<td>Scl-70</td>
<td>0</td>
</tr>
</tbody>
</table>

* Rounded from published literature
Anti-dsDNA in ‘Real Time’ Clinical Practice

Central Triage “CReATe”
Derivation of ANA+ Cohort

Central Triage 3 Year Audit

Total Referrals
N=15,357

Referral Profile

- Inflammatory Arthritis 26%
- Arthralgias 3%
- Autoimmune Disease 12%
- Soft Tissue Rheumatism 3%
- Osteoarthritis 6%
- Other 8%

- Crystal Arthropathy 3%
- Positive ANA/ENA 4.2% (N=643)
- Vasculitis 2%
- Spondyloarthritis 4%

- Foothills Medical Center Evaluation 1.7% (N=263)

Only ~25% of people with a positive ANA were referred
## Demographics ANA Referrals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age /Range (years)</td>
<td>49 / 18 - 86</td>
</tr>
<tr>
<td>% Female</td>
<td>94.1</td>
</tr>
<tr>
<td>% Urban patients: City of Calgary</td>
<td>66.7</td>
</tr>
<tr>
<td>% referred by a family physician*</td>
<td>96.7</td>
</tr>
<tr>
<td>Average wait time (days) from referral date to date seen by rheumatologist</td>
<td>177.8</td>
</tr>
</tbody>
</table>

*an important jurisdictional variable*
Table 2. Autoantibody specificities of 116 patients with positive anti-ENA/dsDNA-ALBIA

<table>
<thead>
<tr>
<th>ENA Autoantibody**</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro52/TRIM21</td>
<td>53</td>
<td>45.7</td>
</tr>
<tr>
<td>SS-A/Ro60</td>
<td>40</td>
<td>34.5</td>
</tr>
<tr>
<td>Chromatin</td>
<td>21</td>
<td>18.1</td>
</tr>
<tr>
<td>SS-B/La</td>
<td>19</td>
<td>16.4</td>
</tr>
<tr>
<td>Topoisomerase I (Scl-70)</td>
<td>17</td>
<td>14.7</td>
</tr>
<tr>
<td>U1 Ribonucleoprotein</td>
<td>17</td>
<td>14.7</td>
</tr>
<tr>
<td>Sm</td>
<td>14</td>
<td>12.1</td>
</tr>
<tr>
<td>Ribosomal P*</td>
<td>10</td>
<td>13.2</td>
</tr>
<tr>
<td>dsDNA*</td>
<td>10</td>
<td>13.2</td>
</tr>
<tr>
<td>Centromere</td>
<td>3</td>
<td>2.6</td>
</tr>
<tr>
<td>Jo-1</td>
<td>3</td>
<td>2.6</td>
</tr>
<tr>
<td>Unidentified</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

3 UCTD, 2 SLE, 2 SjS, 1 SSc, 2 no AARD

Choosing an Anti-dsDNA Assay

• >30 years use of CLIFT but due to clinician demand wanted an assay with higher sensitivity and one that provided quantitative results (clinical follow-up for flares/relapses).

• Three assays compared to 100 CLIFT anti-dsDNA +ve sera
  • ALBIA 68% agreement
  • ELISA 74% agreement
  • CIA 98% agreement
Comparative anti-dsDNA studies*

Infantino et al. Clinical comparison of QUANTA Flash dsDNA chemiluminescent immunoassay with four current assays for the detection of anti-dsDNA autoantibodies.

**J Immunol Res 2015: 902821.**

**Table 3: Clinical performance characteristics for anti-dsDNA antibody assays.**

<table>
<thead>
<tr>
<th></th>
<th>QUANTA Flash dsDNA</th>
<th>QUANTA Lite dsDNA SC</th>
<th>BioPlex 2200 dsDNA</th>
<th>ImmuLisa dsDNA</th>
<th>NOVA Lite dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer’s cut-off used, where equivocal results are considered positive</td>
<td>≥35 IU/mL</td>
<td>≥30 IU/mL</td>
<td>≥5 IU/mL</td>
<td>≥50 IU/mL</td>
<td>N/A</td>
</tr>
<tr>
<td>Sensitivity in SLE% (95% CI)</td>
<td>39.3 (27.2–52.7)</td>
<td>54.1 (40.8–66.9)</td>
<td>44.3 (31.5–57.6)</td>
<td>26.2 (15.8–39.1)</td>
<td>8.2 (2.7–18.1)</td>
</tr>
<tr>
<td>Specificity % (95% CI)</td>
<td>96.0 (90.1–98.9)</td>
<td>91.0 (83.6–95.8)</td>
<td>88.0 (80.0–93.6)</td>
<td>96.0 (90.1–98.9)</td>
<td>100.0 (96.4–100.0)</td>
</tr>
<tr>
<td>LR+</td>
<td>9.84</td>
<td>6.01</td>
<td>3.69</td>
<td>6.56</td>
<td>+∞</td>
</tr>
<tr>
<td>LR−</td>
<td>0.63</td>
<td>0.50</td>
<td>0.63</td>
<td>0.77</td>
<td>0.92</td>
</tr>
<tr>
<td>Odds ratio</td>
<td>15.6</td>
<td>12.0</td>
<td>5.9</td>
<td>8.5</td>
<td>N/A</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.79 (0.72–0.87)</td>
<td>0.90 (0.86–0.95)</td>
<td>0.68 (0.60–0.77)</td>
<td>0.61 (0.52–0.71)</td>
<td>0.54 (0.51–0.58)</td>
</tr>
<tr>
<td>Cut-off used at 94.0% specificity</td>
<td>≥27.5 IU/mL</td>
<td>≥49.6 IU/mL</td>
<td>≥12 IU/mL</td>
<td>≥41.9 IU/mL</td>
<td>N/A</td>
</tr>
<tr>
<td>Sensitivity in SLE% at 94.0% specificity (95% CI)</td>
<td>52.5 (39.3–65.4)</td>
<td>45.9 (33.1–59.2)</td>
<td>21.3 (11.9–33.7)</td>
<td>27.9 (17.1–40.8)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

AUC: area under the curve; LR: likelihood ratio; CI: confidence interval.
QUANTA Flash dsDNA correlates with the Farr RIA

<table>
<thead>
<tr>
<th>Assays</th>
<th>Positive Agreement</th>
<th>Negative Agreement</th>
<th>Total Agreement</th>
<th>Kappa Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>QF (equiv -) vs. Farr</td>
<td>70.4%</td>
<td>78.3%</td>
<td><strong>76.6%</strong></td>
<td>0.41</td>
</tr>
<tr>
<td>QF (equiv +) vs. Farr</td>
<td>80.2%</td>
<td>68.4%</td>
<td><strong>70.9%</strong></td>
<td>0.36</td>
</tr>
</tbody>
</table>

419 consecutive patients
CIA good agreement with Farr assay
CIA better performance than Farr

Toh B-H, et al QUANTA Flash® dsDNA antibody results show close correlation with Farr assay results. Poster 9th International Congress on Autoimmunity, Nice, France, 2014
QUANTA Flash dsDNA differentiates between active and non-active SLE

209 SLE patients
Significant difference in median level between active and inactive SLE

Garcia et al. Strong correlation between QUANTA Flash® anti-dsDNA results and disease activity parameters in systemic lupus erythematosus. Poster on the 9th International Congress on Autoimmunity, Nice, France, 2014
QUANTA Flash dsDNA correlates with the disease activity score SLEDAI

504 SLE patients
Highly significant correlation with SLEDAI

QUANTA Flash dsDNA differentiates SLE

Figure 2  Comparative antibody titer distribution of results among patients with (n=110) and without lupus nephritis (n=195) for a.) QUANTA Flash dsDNA and b.) QUANTA Lite dsDNA SC ELISA. Axes are shown in logarithmic scale for assay units.

Bentow et al. QUANTA Flash dsDNA chemiluminescent immunoassay results show stronger association with lupus nephritis than traditional ELISA. Poster on the 10th International Congress on Autoimmunity, Leipzig, Germany, 2016.
Summary: anti-dsDNA by CIA BioFlash

- Outperforms ALBIA
  - Infantino et al. J Immunol Res. 2015; m902821
  - Fritzler MJ. Internal QA/QC (unpublished)

- High correlation with:
  - CLIFT
    - Infantino JIR 2015; m902821; Fritzler MJ. Intl QA/QC (unpublished)
  - Farr RIA
  - Renal Disease
    - Bentow et al. 10th International Congress on Autoimmunity, Leipzig, Germany, 2016
  - Active disease & SLEDAI
    - Garcia et al 9th International Congress on Autoimmunity, Nice, France, 2014
    - Mahler et al. 3rd International Congress on Controversies in Rheumatology & Autoimmunity (CORA 2015), Naples, Italy, 2015
Which assay should I choose?

Depends on:

A) **Diagnosis in the Clinical Setting**
   - If high pre-test probability high (e.g. specialists with “intent to treat”) then a high specificity but low(er) sensitivity assay may be just fine.
   - If low pre-test probability (e.g. requests from primary care: “case finding”) then a high sensitivity assay may be preferable

B) **Use of test: for prognosis and disease monitoring**
   - Test which predicts risk of more severe disease course (i.e. lupus nephritis, higher SLEDAI)
   - Quantitative test which correlates with (renal) disease activity
Which assay should I choose?

Depends on:

C) Lab requirements

• Automation
  • ELISA/CIA/ALBIA
  • CLIFT on digital automated microscopy (i.e. NOVA View)
• Modern IIF microscope available and skilled staff
• Correlation with methods and/or interface (LIS) already in place
Comments

• In order to deliver useful results of high clinical value a single anti-dsDNA test may not be the ultimate solution
  • Combination of assays that use different DNA sources
  • Combination of a sensitive assay with a specific assay
• BUT: the sensitive assay should not detect low non-specific, low avidity antibodies
• Possible Solution:
  • CIA dsDNA (synthetic) + CLIFT (native)

- Study population 187 SLE (Sweden)
- Assays: CLIFT, FIDIS-ALBIA, Euroline, ELISA-FLIA
- CLIFT: highest specificity (98%)
- When cut-off levels for FIDIS, EliA, and EUROLINE were adjusted according to SLICC-12, specificity of FIDIS comparable to CLIFT.
- FIDIS and CLIFT also showed highest concordance (84%).
- FIDIS performed best regarding association with disease activity in cross-sectional and consecutive samples.
- Conclusion. CLIFT remains a good choice for diagnostic purposes, but FIDIS performs equally well when the cut-off is adjusted according to SLICC-12.
Anti-dsDNA in Inception Cohort of SLE

• 1,137 SLICC patients seen within 6 months of diagnosis

• 66.4% anti-dsDNA positive by CIA (conventional cut-off)

• ~50% anti-dsDNA using SLICC-12 cut-off

Why is that important?

• Should anti-dsDNA always be associated with homogenous IIF pattern?

• Should it correlate with SLEDAI or renal disease?
  • 31.6% had evidence of renal disease at first visit

Future Considerations

• Despite over half a century of anti-DNA research, still no “gold standard”.
• More studies of real time assay performance are required
• ACR and SLICC Criteria review. Is a single +ve test sufficient? Which assay(s)? What is the cut-off?
• Which assay(s) have the highest predictive value?
  • Longitudinal studies of UCTD needed
• Which assay(s) should be used for enrollment into clinical trials?
• What are we actually measuring in the anti-dsDNA assay?
  • Cationic proteins secondarily binding to DNA (histones, C1q, lactoferrin, etc.)
  • Detailed dsDNA/anti-dsDNA proteome needed
• What about Circular RNA? A recent “hot” topic
The Bigger anti-DNA Picture
Acknowledgements

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Eliminating Health Disparities in Lupus
THANK YOU
Inova Diagnostics/Werfen Australia

CONNECT COLLABORATE INNOVATE
OMEGA — Ω
Choi MY, Barber MRW, Barber CEH, Clarke AE, Fritzler MJ. *Preventing the development of SLE: Identifying risk factors and proposing pathways for clinical care.* Invited submission Lupus 2016.
# IIF Patterns of ANA+

<table>
<thead>
<tr>
<th>ANA IIF Pattern</th>
<th>%</th>
<th>Titer Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speckled</td>
<td>60%</td>
<td>1/160-1/5120</td>
</tr>
<tr>
<td>Nucleolar</td>
<td>25%</td>
<td>1/160-1/5120</td>
</tr>
<tr>
<td>Cytoplasmic Speckled</td>
<td>27%</td>
<td>1/160-1/5120</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>21%</td>
<td>1/160-1/5120</td>
</tr>
<tr>
<td>Dense Fine Speckled</td>
<td>10%*</td>
<td>1/160-1/5120</td>
</tr>
<tr>
<td>Other</td>
<td>37%</td>
<td>1/160-1/5120</td>
</tr>
</tbody>
</table>

(*25/245)