"EVERYTHING YOU DIDN'T WANT TO KNOW ABOUT ANTI-DNA, AND MORE

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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.

Advanced Diagnostics Laboratory Mitogen

OUTLINE

- Historical Perspective:
  - The Discovery of anti-DNA antibodies.
  - Anti-dsDNA as classification/diagnostic criteria
- An enigma wrapped in a paradox: Patient Presentation
  - ANA negative/anti-dsDNA positive
- Origins + drivers of the B Cell Response
  - The Spectrum of anti-DNA antibodies
- Immunoassays Used to Detect anti-dsDNA: What are they actually measuring?
- The Main Clinical Applications and Uses
- Where should research focus in the future?

What is anti-DNA?

Bev Doolittle "Red Fox"

Good News & Bad News: The Kodachrome Legacy

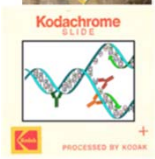
First the bad news

- 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

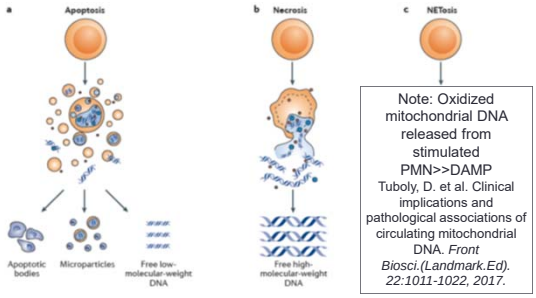
- 1957: Holman & Kunkel show that DNase of deoxyribonucleoprotein (DNP) eliminated the LE cell. ***Science 126: 162-3, 1957***
- 1966: Tan, Schur, Carr et al. DNA and anti-DNA in SLE ***J Clin Invest 45: 1732-40, 1966***
- 1967: Koffler, Schur & Kunkel elute anti-DNA from SLE kidney. ***J Exp Med 126: 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***

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

D. S. Pisetsky. Anti-DNA antibodies - quintessential biomarkers of SLE. *Nat Rev Rheumatol 12:102-110, 2016.*

The "Drivers" of anti-DNA Responses



Note: Oxidized mitochondrial DNA released from stimulated PMN>>DAMP
Tuboly, D. et al. Clinical implications and pathological associations of circulating mitochondrial DNA. *Front Biosci.(Landmark.Ed). 22:1011-1022, 2017.*

D. S. Pisetsky. Anti-DNA antibodies - quintessential biomarkers of SLE. *Nat Rev Rheumatol 12:102-110, 2016.*

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/formed *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".

* See Rekvig O. Clin Exp Immunol 179:75, 2014.

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?

Antecedent Factors: UCTD Evolving to SLE

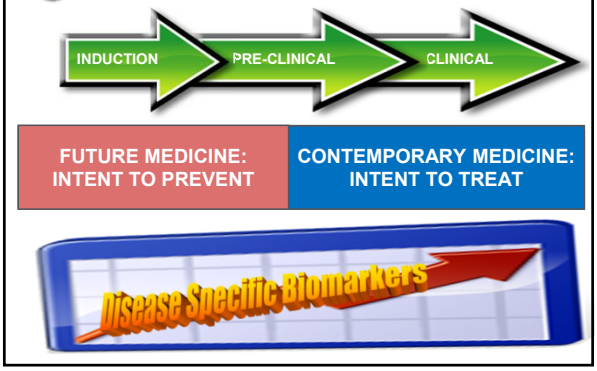
CLINICAL

- Fever
- Discoid lupus
- Serositis
- Photosensitivity
- Leukopenia

SEROLOGY

- **Homogeneous ANA**
- **Anti-dsDNA**
- Anti-Sm
- Anti-cardiolipin
- Multiple SLE-related autoantibodies

TIME COURSE SLE & AARD



Autoantibody Profile of UCTD evolving to SLE

AUTOANTIBODY	RANGE %*
ANA	60 - 100
dsDNA	5 - 20
SSA/Ro60	10 – 30
SSB/La	0 – 5
Sm	0 – 5
U1RNP	0 - 30
Scl-70	0

* Rounded from published literature

ACR Classification Criteria 1977 Update

10. Immunologic Disorder	1. Anti-DNA: antibody to native DNA in abnormal titer 1. OR 2. Anti-Sm: presence of antibody to Sm nuclear antigen 1. OR 3. Positive finding of antiphospholipid antibodies on: 1. 1. an abnormal serum level of IgG or IgM anticardiolipin antibodies, 2. 2. a positive test result for lupus anticoagulant using a standard method, or 3. 3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test

No detection method mentioned

1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus. http://www.rheumatology.org/Practice-Quality/Clinical-Support/Criteria/ACR-Endorsed-Criteria

SLICC Criteria 2012

Immunological Criteria

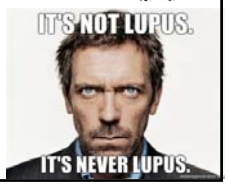
1. ANA above laboratory reference range
2. Anti-dsDNA above laboratory reference range, except ELISA: twice above laboratory reference range
3. Anti-Sm
4. Antiphospholipid antibody: any of the following
   - lupus anticoagulant
   - false-positive RPR
   - medium or high titer anticardiolipin (IgA, IgG or IgM)
   - anti-β_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*

Detection method AND specific thresholds

Petri et al. Derivation and Validation of Systemic Lupus International Collaborating Clinics Classification Criteria for Systemic Lupus Erythematosus. Arthritis Rheum 64(8): 2677–2686, 2012.

Is *this* lupus…

- A 69-year-old female presented with month history of symmetric polyarthritis, pleuritis and weight loss (10kg)
- Initial laboratory analysis:
  - Lymphopenia 0.3 x 10^9/L
  - Elevated ESR 48mm/hr, CRP 93.0mg/mL
  - ANA was negative by IIF on HEp-2 cells
  - **BUT….high titre anti-dsDNA**



Positive dsDNA but "negative" ANA?

Antibody Profile	ANA		dsDNA			ENA		Chromatin	C1q	CCP	RF	SSc Panel	ILD/ Myositis Panel
Assay (Units)	HEp-2 (dil: 1/80)	CLS (1/80)	CIA (IU/mL)	Quanta LITE (AU)	Crithidia luciliae (dil 1:20)	MagPix (CU)	Euroline (DU)	ELISA (Inova)	ELISA (Inova)	ELISA (Inova)	(kU/L)	Euroline LIA	Euroline LIA
Result	Cytoplasmic speckled 1/160	Negative	Positive (667)	Positive (399.2)	Positive (1/320)	dsDNA (419)	dsDNA (28)	Negative	Negative	Negative	Negative	Negative	Mi-2 positive SRP weak positive

"Negative" ANA or positive cytoplasmic speckled ACA. Confirmed on 3 different assays: Hep-2 Inova, Hep-2 NOVA View, and Euroimmun

Nuclear homogenous IIF appears with 0.1N HCl protein extraction

Positive dsDNA but "negative" ANA?

- High titre anti-dsDNA but a negative ANA IIF can be observed in up to 1/150 anti-dsDNA sera
- Hidden dsDNA epitopes via proteins (e.g. histones) may be missed by the IIF ANA test

How common is ANA negative SLE?

CONCISE REPORT

Assay variation in the detection of antinuclear antibodies in the sera of patients with established SLE

David S Pisetsky,[1] Diane M Spencer,[1] Peter E Lipsky,[2] Brad H Rovin[3]

Ann Rheum Dis 2018; **Epub ahead of print,** doi:10.1136/annrheumdis-2017-212599

Table 2 Antinuclear antibody testing kits

Test result	IFA kit 1	IFA kit 2	IFA kit 3	ELISA	Multiplex
Negative	23 (22.3)	10 (9.7)	5 (4.9)	12 (11.7)	14 (13.6)
Indeterminate	9 (8.7)	10 (9.7)	2 (1.9)	0	8 (7.8)

Key points:
- Serum dilution 1/40
- Only nuclear staining regarded as positive

Anti-dsDNA in an Inception Cohort of SLE

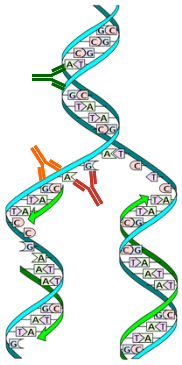
- **1,137 SLICC patients seen within 6 months of diagnosis**
- 66.4% anti-dsDNA positive by CIA (conventional cut-off)
- ~50% anti-dsDNA using SLICC-12 cut-off
- 71/1137 (6.2%) were ANA-negative,
- notably 11.3% of anti-dsDNA +ve had NEGATIVE ANA **Why is that important?**
- Anti-dsDNA is not always associated with homogenous IIF pattern…or any pattern at all on HEp-2
- Should it correlate with SLEDAI or renal disease?
  - 31.6% had evidence of renal disease at first visit

Choi MY, et al. Arthritis Care & Res in revision 2018)

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, elongated
  - **Triplex**

THE Reference: Stollar BD Molecular analysis of anti-DNA antibodies. FASEB J 8:337, 1994

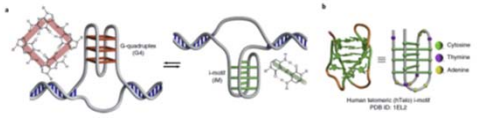


nature chemistry ARTICLES

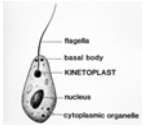
I-motif DNA structures are formed in the nuclei of human cells

Mahdi Zeraati[1,2], David B. Langley[1], Peter Schofield[1], Aaron L. Moye[1], Romain Rouet[1], William E. Hughes[1,3], Tracy M. Bryan[1], Marcel E. Dinger[1,2,*] and Daniel Christ[1,2,*]

- "4-stranded" DNA



- Found in:
  - Telomeres (think NOR-90/hUBF)
  - Centromeres
  - Promotor regions of oncogenes
  - negative superhelices induced during transcription
  - "Molecular Crowding" (think Crithidia kinetoplast)



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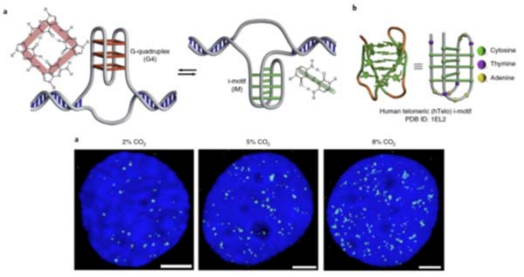
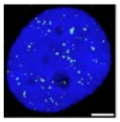
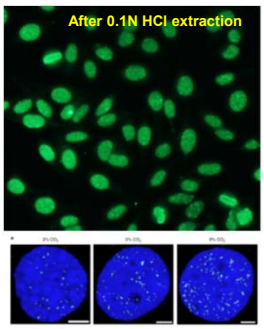


Another SLE serum
ANA –ve/dsDNA+ve





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 Immunoassya (ALBIA): Luminex platform
  - Chemiluminescence immunoassays (CIA): BioFLash platform
- IIF assays
  - Crithidia lucilliae (CLIFT)

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

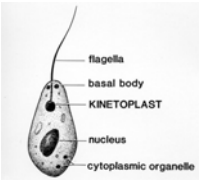
Take home message regarding the antigen source is:

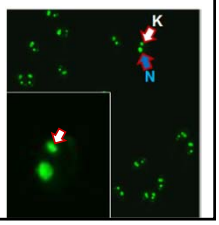
- Source should have no ssDNA, histone, phospholipid or other contamination.
- **AND**
  pure dsDNA is unlikely to occur in nature, hence:
- "Testing for anti-dsDNA antibodies using pure dsDNA as target antigen is, by definition, an artificial analytical approach." (Rekvig O, Clin Exp Immunol 179:75, 2014)
- Is DNA **sequence a factor...maybe?**

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.
  Am.J.Gastroenterol. 104:1420, 2009.

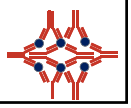
Nguyen Swain, Norman, Fritzler replicated findings BUT not anti-dsDNA +ve in other immunoassay PLoS.ONE. 13:e0193960, 2018.





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 more common than most believe.



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**

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Summary: anti-dsDNA by CIA

- **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**
    - Toh B-H., et al 9th International Congress on Autoimmunity, Nice, France, 2014
  - **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 is the 'best' ?

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 is the best?

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)**

Enocsson, C. et al.. Four Anti-dsDNA Antibody Assays in Relation to Systemic Lupus Erythematosus Dis… Specificity and Activity. *J.Rheumatol. 42:81…*

- Study population 187 SLE (Sweden)
- **Assays: CLIFT, FIDIS-ALBIA, …**
- CLIFT: highest specificity (…
- When cut-off levels for FI… …ding to SLICC-12 (i.e. do… …of FIDIS comparable to C…
- FIDIS and… …84%).
- FIDI… …ease activity in cross-… …garding association with certain …
- **C… …mains a good choice for diagnostic pu… …DIS performs equally well when the cut-off is ac… …cording to SLICC-12.**

A QUICK COMMENT ON SLICC-12 AND CUT-OFFS

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: what will new ACR/EULAR criterion be?
- 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 mitochondrial and I-motif DNA?**

The Bigger anti-DNA Picture

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