Autoantibodies giving rise to cytoplasmic IIF staining using HEp-2 cell substrate

Some associations of anticytoplasmic antibodies with clinical diagnoses and features

HEp-2 IIF: the gold standard for ANA screening.
Meroni PL, Schur PH: an old test with new recommendations.
Proposed taxonomy of HEp-2 cell staining patterns elaborated in the EU CANTOR project 1998-2000

Membranous nuclear patterns:
- Smooth membranous nuclear
- Punctate membranous nuclear

Nucleoplasmic patterns:
- Homogeneous nucleoplasmic
- Large speckled nucleoplasmic
- Coarse speckled nucleoplasmic
- Fine speckled nucleoplasmic
- Fine grainy nucleoplasmic
- Pleomorphic speckled (PCNA)
- Centromere
- Multiple nuclear dots
- Coiled bodies (few nuclear dots)

Nucleolar patterns:
- Homogeneous nucleolar
- Clumpy nucleolar
- Punctate nucleolar

Spindle apparatus patterns:
- Centriole (centrosome)
- Spindle pole (NuMa)(MSA-1)
- Spindle fibre
- Midbody (MSA-2)
- CENP-F (MSA-3)

Cytoplasmic patterns:
- Diffuse cytoplasmic
- Fine speckled cytoplasmic
- Mitochondrial-like
- Lysosomal-like
- Golgi
- Contact proteins
- Vimentin-like

Negative
Undeterminable

Wiik A. et al. J.Autoimmun. 2010
IIF staining patterns on HEp-2 cell substrate.

Membranous nuclear patterns:
- Smooth membranous nuclear
- Punctate membranous nuclear

Nucleoplasmic patterns:
- Homogeneous nucleoplasmic pattern
- Large speckled nucleoplasmic
- Coarse speckled nucleoplasmic
- Fine speckled nucleoplasmic
- Fine grainy Scl-70 like nucleoplasmic
- Pleomorphic speckled (anti-PCNA)
- Centromere
- Multiple nuclear dots
- Coiled bodies (few nuclear dots)

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- Cytoplasmic
- Mitochondrial-like
- Lysosomal-like
- Golgi-like
- Contact proteins
- Vimentin-like
- Negative
- Undeterminable

All of these have clinical associations with some diagnosis and/or features!
Advantages of HEp-2 cell IIF

- **Intact permeable cells** contain the relevant autoantigens *in situ* in resting cells and cells at different stages of division. But the reactivity with autoantibodies depends on whether the right conformational state of the antigen has been preserved. The way the cells are fixed is very important! The **batch** is important.

- Morphological recognition of HEp-2 cell staining patterns using one good HEp-2 cell substrate is a natural talent of many people: The European multicenter study (CANTOR) proved that!

- Note: Autoantigen mixtures coated on solid phase supports (ELISA plates, beads, arrays etc.) cannot substitute for the HEp-2 IIF test since many autoantigens do not react with their corresponding antigen (low concentration, denaturation etc.)
Indirect immunofluorescence

- Fluorochrome conjugate
- "ANA"
- HEp-2 cell

Very sensitive
Broad screening potential

Clinically meaningful cut-off setting is crucial!

How do you determine such cut-off?
Indirect immunofluorescence

- Very sensitive
- Broad screening potential

Fluorescence

- Fluorochrome conjugate
- "ANA"
- HEp-2 cell

Clinically meaningful cut-off setting is crucial!

How do you determine such cut-off?

Most laboratories investigate sera from healthy donors and set the borderline at max. 5% positivity
Control line: IgG

SmB
SmD
RNP-70
RNP-A
RNP-C
Ro 52
SSA/Ro 60
SSB/La
CENP-B
Scl-70
Jo-1
Ribo P
Histones

Conjugate

Serum + conjugate

How do you set cut-off here?
Line immuno-assay

The borderline for positivity of each antibody has been set using sera from differential diagnostic rheumatic disease populations of patients but also healthy control sera.
From ANA pattern to specific antibody testing: Example - Homogeneous nucleoplasmic pattern

SLE, RA, JCA

Chromatin constituents: dsDNA, histones, nucleosomes, HMGs

Reflex tests

Lab.test:

Anti-dsDNA
- Farr assay, Crithidia IF ELISA Line IA
- Anti-histone ELISA Line IA
- Anti-nucleosome ELISA Line IA
- Anti-HMG
  - At present: no agreed assay

Note that the choice of assay technique for anti-dsDNA determination has a strong influence on the value for clinical interpretation and
Cytoplasmic autoantibodies to-

Nucleus

- Centrioles
- Spindle pole
- Spindle fibres
- Midbodies
- CENP-F
- Ribosomes
- Mitochondria
- Lysosomes
- Early endosomes
- GW bodies
- Proteasomes
- Exosomes
- Golgi apparatus
- F-Actin
- Contact proteins
- Vimentin
Centrosome (centriole)

Scleroderma, Raynaud’s syndrome

No commonly used reflex test: $\alpha/\gamma$–enolase, pericentrin, ninein, Cep-250, Mob1, PCM-1/-2
Spindle fibre

SLE, Sjögrens syndrome

No commonly used reflex test: HsEg5
Spindle pole: Anti-NuMa (MSA-1)

Mycoplasma pneumoniae infections

No commonly used reflex test: centrophilins, NuMa, SP-H
Anti-Midbody (MSA-2)

Scleroderma, Raynaud’s syndrome

No reflex test
CENP-F pattern (MSA-3)

Malignancies (breast, lung, NHL, > 50 %.)

Note zipper-like staining

No reflex test

Note different staining intensity
Ribosomal P-protein

SLE, lupus nephritis, CNS lupus

Several reflex tests
Ribosomal P-protein

About 20 – 30 % of sera are negative by IIF test

SLE, lupus nephritis, CNS lupus

Several reflex tests
Ribosomal RNA & P-protein

SLE

RNA precipitation assay
Anti-ribosomal P antibodies: variety of IIF patterns using different HEP-2 assays.

3 "monospecific" sera.

CDC no.12

3 different HEP-2 assays
Anti-Jo-1: fine speckled or diffuse cytoplasmic

Several reflex tests available. Note that any amino-acyl tRNA synthetase antibody can give the same staining pattern.

Dermatomyositis, polymyositis, interstitial lung disease
Anti-Jo-1: fine speckled or diffuse cytoplasmic

Only about 20-30% of anti-Jo-1 pos. sera are pos by IIF
Anti-aminoacyl-tRNA antibodies

- Histidyl tRNA synthetase: Jo-1
- Threonyl tRNA synthetase: PL-7
- Alanyl tRNA synthetase: PL-12
- Isoleucyl tRNA synthetase: OJ
- Glycyl tRNA synthetase: EJ
- Lysyl tRNA synthetase: SC
- Asparaginyl tRNA synthetase: KS
- Leucyl tRNA synthetase:
- Glutaminyl tRNA synthetase

Anti-synthetase syndrome markers
Signal recognitions particles (SRP)

Necrotizing polymyositis

Reflex test: purified or recombinant SRP-54
Human exosome antibodies: C1D is part of the PM/Scl complex

No reflex test: C1D

Polymyositis/Scleroderma overlap (23%)
Mitochondrial-like

Primary biliary cirrhosis

No reflex tests necessary: 2-oxo acid dehydrogenase family enzymes.
Peroxisomes
Early endosomes

Subac. cutan.
lupusRA, SLE,
Raynaud’s syndr.
Neurolog. disease

No reflex test:
EEA-1

AW 2012
Anti-G(glycine)W(tryptofan) bodies

**Figura 1** – Hep2000 cells stained with patient serum containing autoantibodies to GWBs (red). The nuclei are stained with DAPI (blue). Arrows are pointing to different sized GWBs while the arrowhead marks a cell undergoing mitosis.
GW bodies: RNA processing bodies

neurological diseases (ataxia, motor/sensor neuropathies), SjS, PBC

No reflex tests available: Ge/Hedls, GW182, Ago2
GW body functions

- siRNA function:
  - Jakymiw et al., 2005
  - Liu et al., 2005

- miRNA silencing:
  - Liu et al., 2005
  - Rehwinkel et al., 2005
  - O'Donnell et al., 2005
  - Hatfield et al., 2005

- mRNA storage:
  - Eystathioy et al., 2002
  - Brengues et al., 2005

- mRNA degradation:
  - Eystathioy et al., 2003
  - Sheth & Parker, 2003
  - Cougot et al., 2004

- Cell cycle:
  - Yang et al., 2004

- Cell proliferation:
  - Yang et al., 2004

GW body

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Cytoplasmic discrete speckles (CDS): Ab. To diacyl-phophstidylethanolamine
Cytoplasmic discrete speckles (CDS): Ab. to diacyl-phophstidylethanolamine

Systemic and organ-specific autoimmune disorders.
Anti-mitochondrial and anti-GWB antibodies in 1. biliary cirrhosis

Mitochondrial  GW Bodies  Fusion image

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"Lysosomal-like" pattern

Reflex tests not yet available
Early endomes, GW bodies, lysosomes?
Early endosomes, lysosomes, GW bodies?
Golgi apparatus

SjS, SLE, RA, ataxia, viral infections

Reflex tests not yet available. Most common antigens are giantin and golgin245
Golgi apparatus
Figure 2. Cytoplasmatic rod- and ring-shaped pattern Apparently associated with HCV infection and interferon treatment. Target antigens of these antibodies are under definition.
Induction by CTP and GTP inhibition
Intermediate structures of rods and rings

Courtesy of EKL Chan, Gainesville, Florida
Actin-like

Chronic active hepatitis

Reflex test: F-actin ELISA
Tropomyosin
Contact fibres: tropomyosins?

Ulcerative colitis?
Behcet’s disease?
Vimentin-like
How can you ensure the quality of your HEP-2 cell substrate?

- Establish a collection of 20 – 30 sera, most of which are IIF ANA- or cyto-positive (+ - ++++)
- Run these sera at a fixed dilution every time you order a new batch of slides (on your present batch and the new batch).
- Compare the patterns seen with your present batch and demand that the same patterns are seen with the new batch.
- Stay with slides from a provider/company that you are familiar and satisfied with.
”False positive” is often used

- When found out of commonly recognized clinical context (other disease) and in cases that have not yet presented typical signs of disease (pre-disease, hidden disease, early disease,).
- When a pos. IIF pattern is unknown to the reader/receiver, has not been given an appropriate and agreed name, and when disease associations are unknown.
- When borderline has been set too low.
”False negative” is used

- When IIF HEP-2 is negative (HEp-2 autoantigen not well preserved) though an autoantibody of clinical significance can be detected by other technique. Some examples: anti-Jo1, -Ro 60, -ribo P
- When borderline for positivity of the IIF HEP-2 test has been set too high.
- When borderline for specific autoantibody detection (ELISA etc.) has been set too low.
- When the laboratory decides to report a ”neg. IIF ANA” result although staining was actually seen. The positive result is thus not reported.
What are the clinical aspects?

- Some ANA have well-known clinical associations, but the target antigen specificity needs to be revealed by techniques other than IIF (ELISA, bead assays, chip assays, immunodiffusion etc).

- Some ANA have less clear-cut clinical utility, mainly because only modest efforts have been spent to harmonize their recognition by IIF and study their antigen specificity by independent techniques, and thus sufficiently large populations of patients have not been available for detailed clinical analyses.

- Some ANA are rare [”esoteric”] (<5%) and thus have not been focused on because they were considered clinically ”insignificant” although there is no basis for this assumption.

- The present concept is that all ANA have clinically significant associations when large patient cohorts are studied, but that demands set up of co-ordinated multi-centre studies.
Autoantibody conundrum: clinical value of esoteric autoantibodies.

Studies of disease cohorts indicate low frequency (<5%) of antibodies to CENP-F, PCNA, NuMA, HsEG5, GW bodies, Golgi, early endosomes (EEA-1), PML bodies, coiled bodies.

But-

Studies of serological cohorts of positive sera show a high frequency of certain autoimmune syndromes e.g.:
Antibodies to PCNA, NuMA, HsEG5, GW, Golgi, EEA-1 indicate presence of SLE or SjS.
PML antibodies indicate PBC in 35% of cases!
Anti-CENP-F indicates malignancies in 50-80% of cases!
Antibodies to Golgi, GW bodies, early endosomes indicate autoimmune neurological disease in a high percentage of cases.
Conclusions.

- Until further the IIF HEp-2 cell screen technique is the gold standard for detection of non-organ specific autoantibodies.
- Carefull cut-off setting for pos. ANA is crucial for diagnostic use and for further work-up.
- No pos. reaction can *á priori* be dis-regarded as meaningless for the clinic. Large studies needed.
- A neg. reaction on one HEp-2 cell substrate can be found to be pos. using another substrate.
- Discuss with the lab. what should be reported to the clinic.
Conclusions.

- Discuss with the lab. whether comments should be added on reports of pos. results.
- ”False pos.” and ”false neg.” have never been defined and agreed upon by consensus.
- The same mono-specific serum may give rise to more than one IIF pattern using different HEp-2 cell substrates.
- The use of an agreed HEp-2 IIF atlas for pattern classification is strongly advised. Free for use is [www.percepton.com/wisecase/](http://www.percepton.com/wisecase/)
  Look at download/documents/atlas
Clinical and serological evaluation of a novel CENP-A peptide based ELISA

Receiver operating characteristics analysis. Receiver operating characteristics (ROC) analysis was performed using the data derived from all centres. Cut-off value of 1.5 RU is indicated by the arrows. ROC curve is shown in a) and ROC decision plot is shown in b) for the sensitivity and specificity. Mahler M et al. AR+T 2009
ROC plot and ROC decision plot. Example: anti-CENP-A ELISA

Mahler M et al. AR+T 2010.