Autoimmune Disease: So Many Tests, But Not So Complicated

Gary L. Horowitz, MD
Beth Israel Deaconess Medical Center
Boston, MA

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

Autoantibodies We Will Cover

Method Used	Abbreviation	Full Name
Indirect Immuno-fluorescence	ANCA	Anti-Neutrophilic Cytoplasmic Antibody
	ANA	Anti-Nuclear Antibody
	AMA	Anti-Mitochondrial Antibody
	ASMA	Anti-Smooth Muscle Antibody
	APCA	Anti-Parietal Cell Antibody
"ELISA"	anti-TTG	Anti-Tissue Transglutaminase Antibody
	anti-DGP	Anti-Deamidated Gliadin Peptide
	anti-TPO	Anti-Thyroid Peroxidase Antibody
	anti-Tg	Anti-Thyroglobulin Antibody
	RF	Rheumatoid Factor
	anti-CCP	Anti-Cyclic Citrullinated Antibody

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

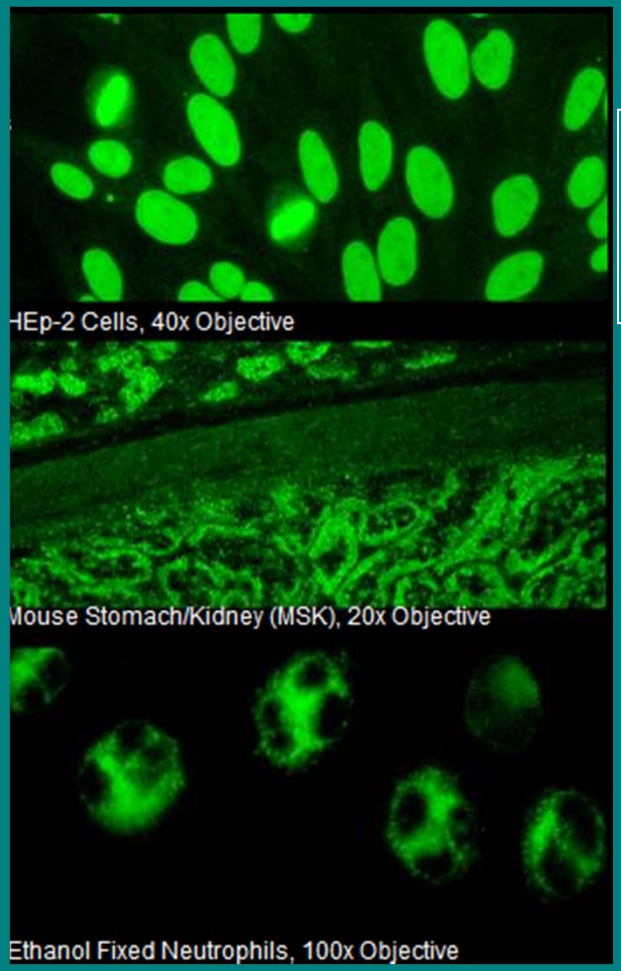
- After reading more about the test online, she infers that she must have SLE (Systemic Lupus Erythematosis) and envisions a very poor prognosis
- She is not scheduled to see the rheumatologist in follow-up for two weeks

Methods in Use

- Indirect Immunofluorescence

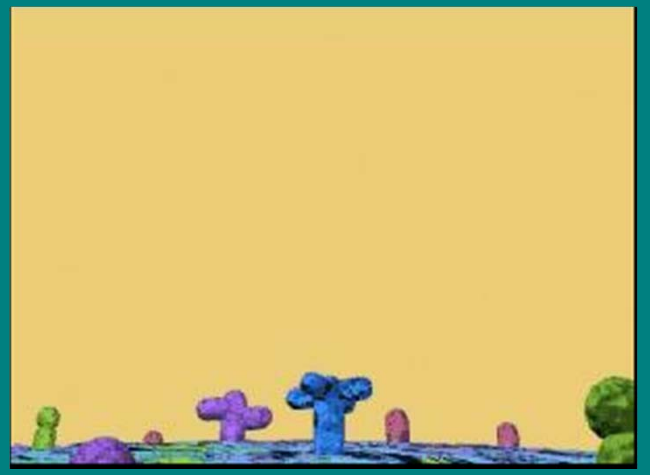
- Immunoassays
  - Standard ELISAs
  - “Multiplex” Immunoasssays

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

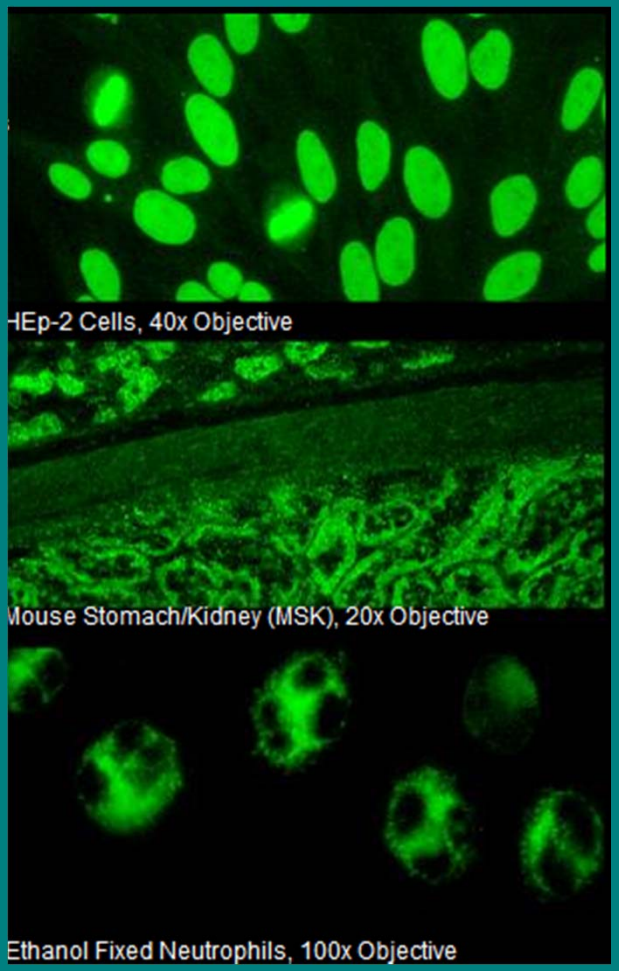


multiple well slides; different cell types on different slides

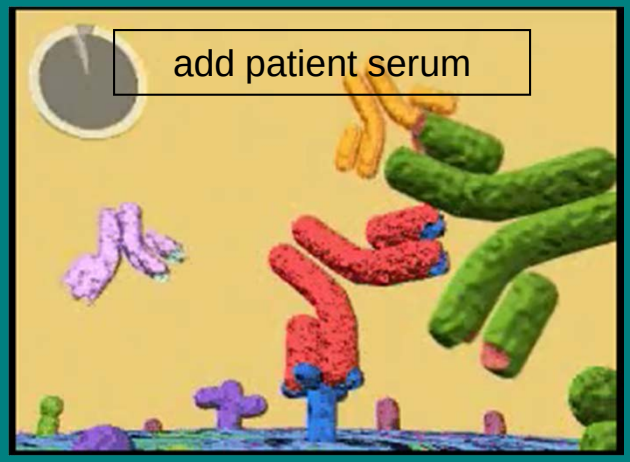
each well contains thousands of antigens

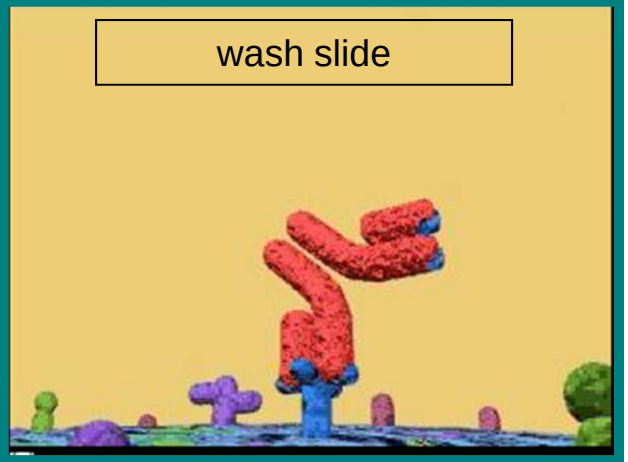


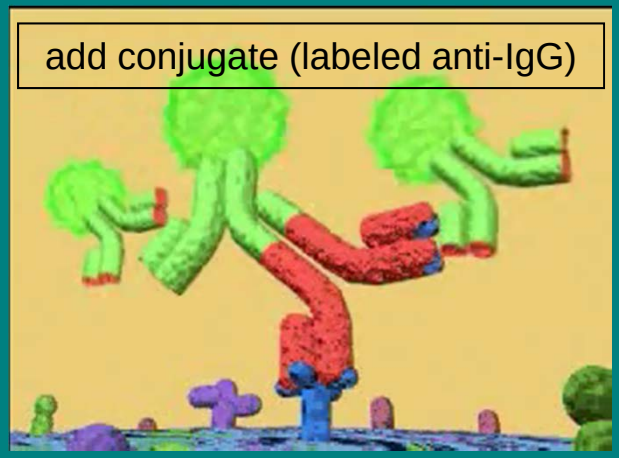
Adapted from Bio-Rad AutoimmuneTUTOR

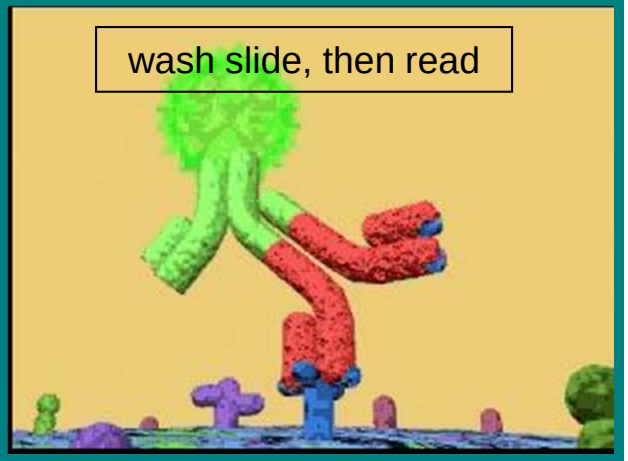


Adapted from Bio-Rad AutoimmuneTUTOR

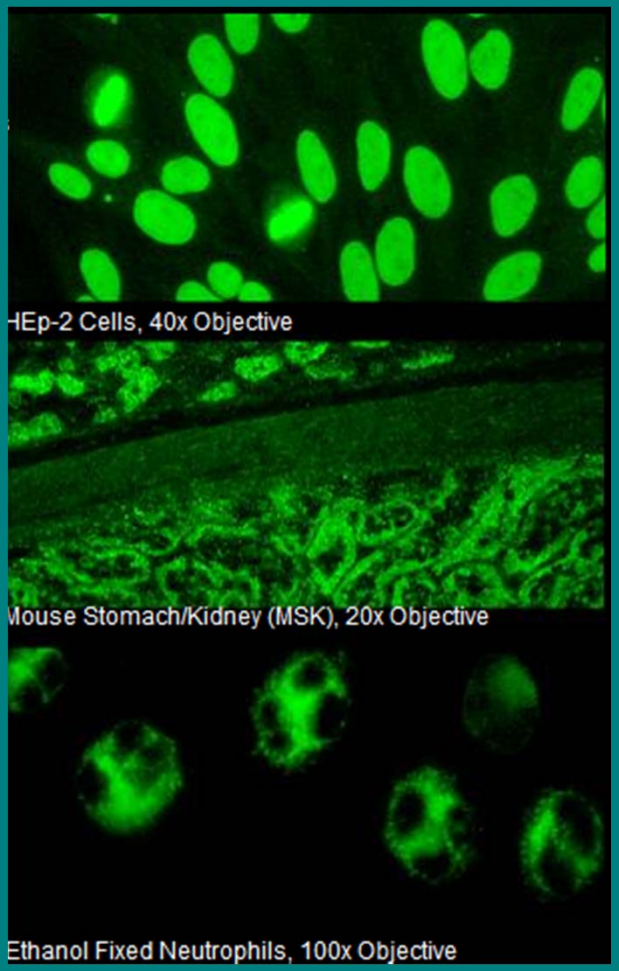








Substrate Specificity



Adapted from Bio-Rad AutoimmuneTUTOR

HEp2 Cells: Anti-Nuclear Antibodies (ANA)

Mouse Stomach/Kidney Cells:
- Anti-Mitochondrial Antibodies (AMA)
- Anti-Smooth Muscle Antibodies (ASMA)
- Anti-Parietal Cell Antibodies (APCA)

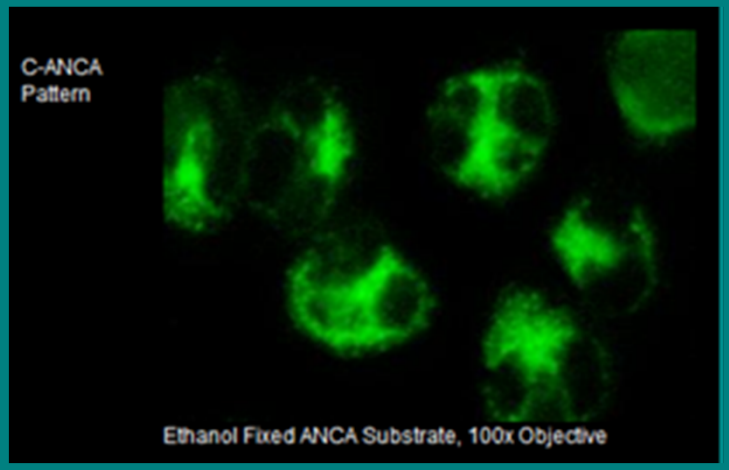
Neutrophils
- Anti-Neutrophil Cytoplasmic Antibodies (ANCA)

Immunofluorescence Technique Notes

- Using different substrates (cell types),
  one “captures” antibodies with different specificities

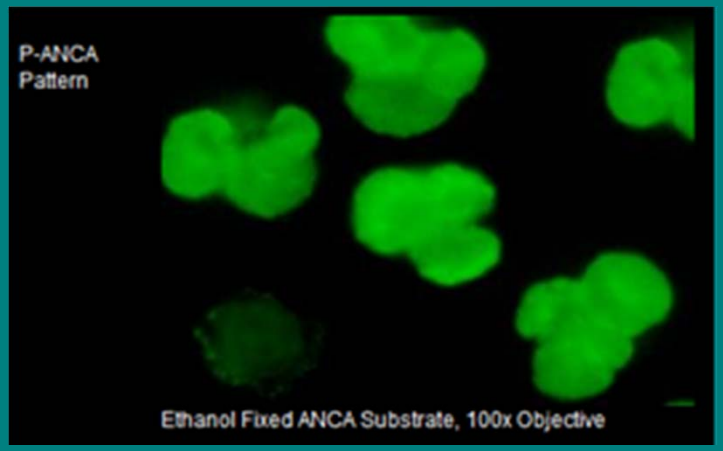
- In some cases, the “pattern” of staining may
  be helpful, suggesting one disease over another

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

Adapted from Bio-Rad AutoimmuneTUTOR

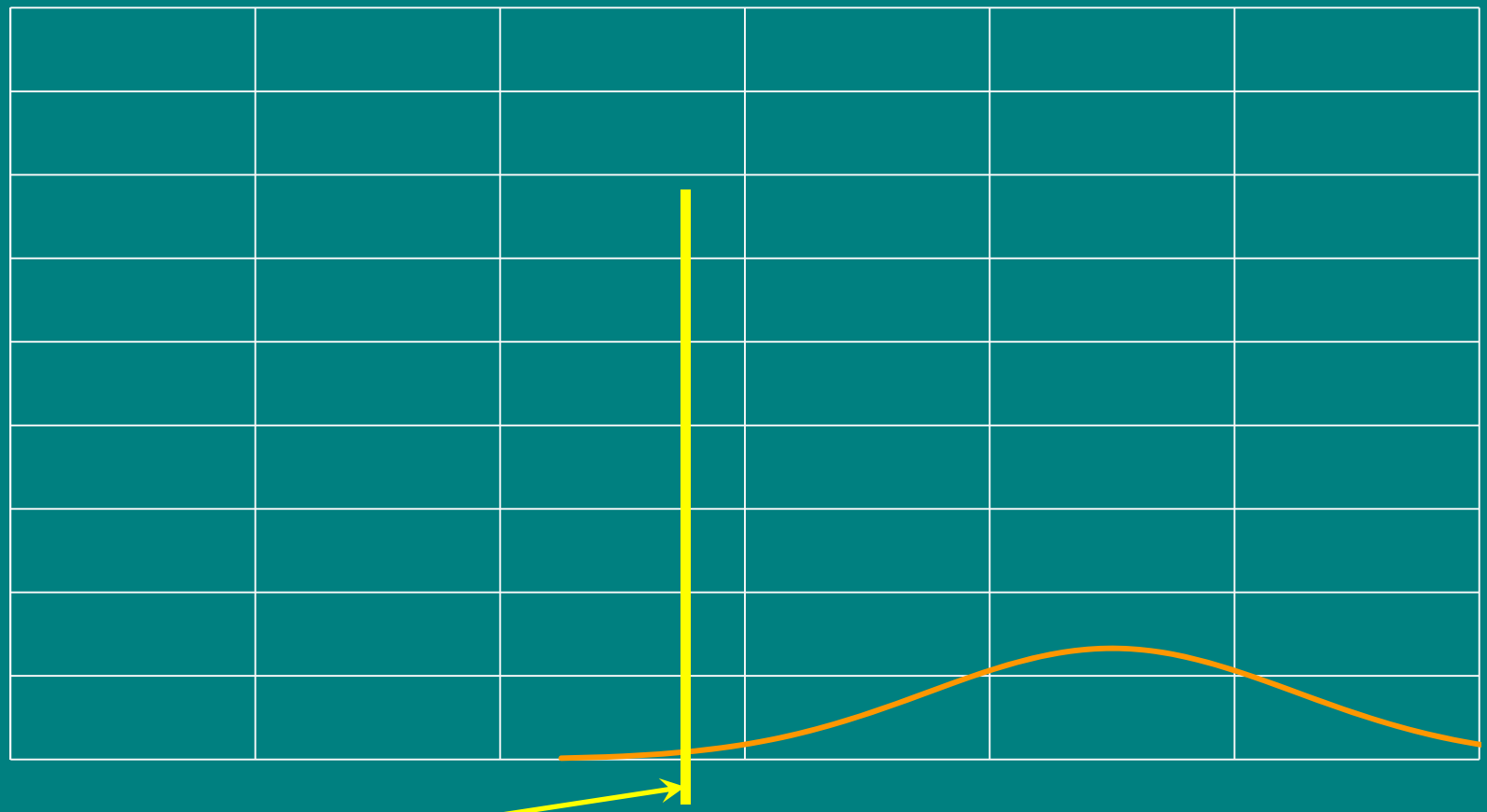
Immunofluorescence Technique Notes

- 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
- 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.

Immunofluorescence Technique Notes

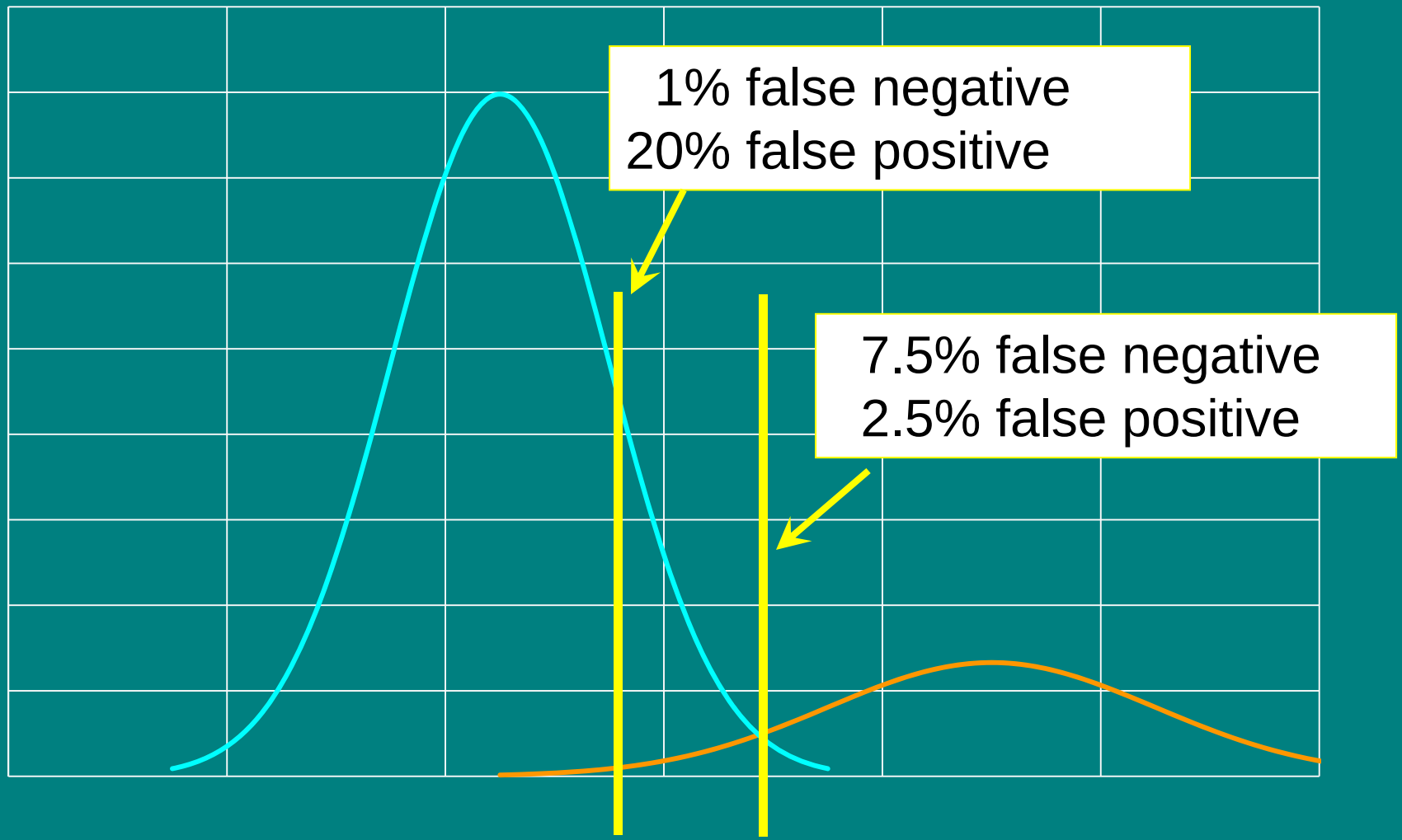
- Using different substrates (cell types),
  one “captures” antibodies with different specificities
- In some cases, the “pattern” of staining may
  be helpful, suggesting one disease over another
- Quantitation is crude (by clinical chemistry standards):
  - make serial dilutions until the result is negative
- Autoantibodies occur frequently in healthy individuals
  - what distinguishes disease from normal is “titer”

Distribution of Values from Patients with Disease



cut-off that detects 99% of patients

Add Distribution of Values from Healthy People



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

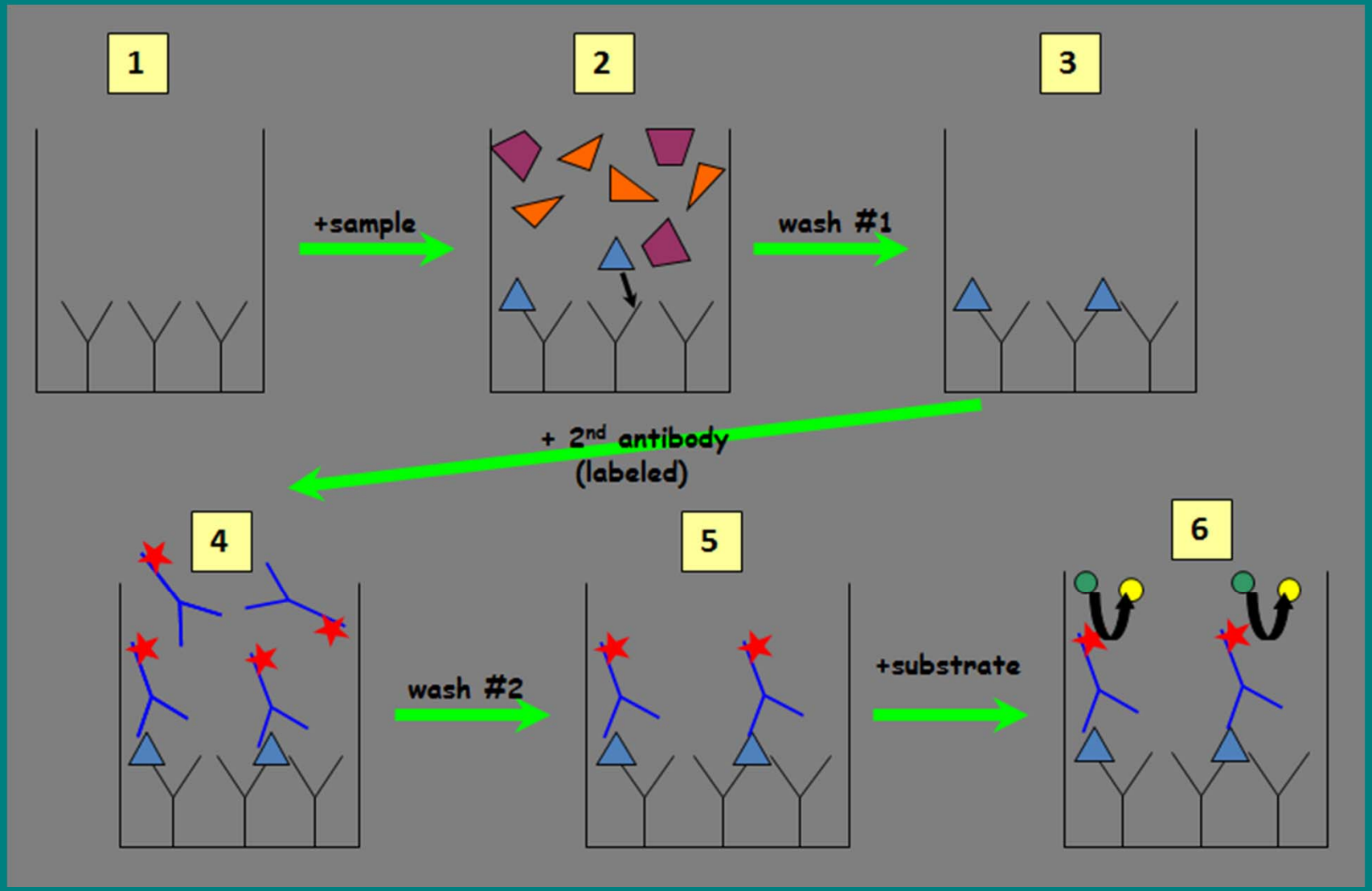
- ANA was “positive” at 1:40 titer
- History was not suggestive of SLE (or any other “collagen vascular disease”)

- 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

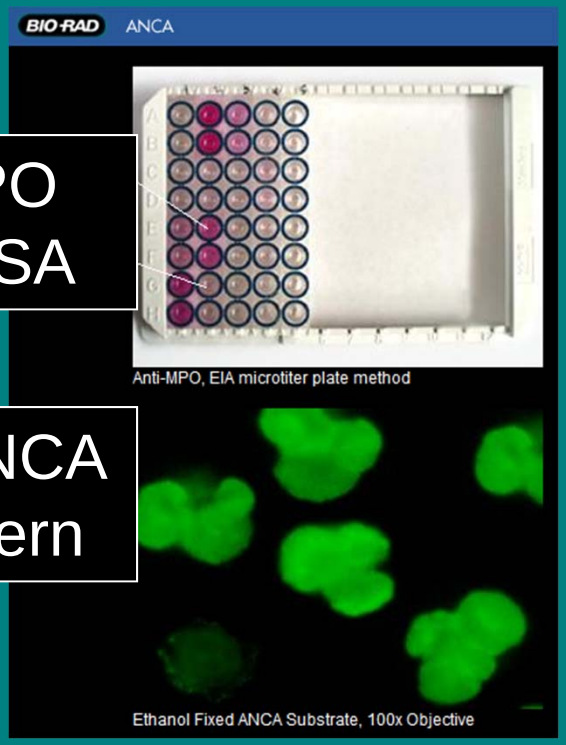
- Indirect Immunofluorescence
- Immunoassays
  - Standard ELISAs
  - “Multiplex” Immunoasssays

ELISA Technique



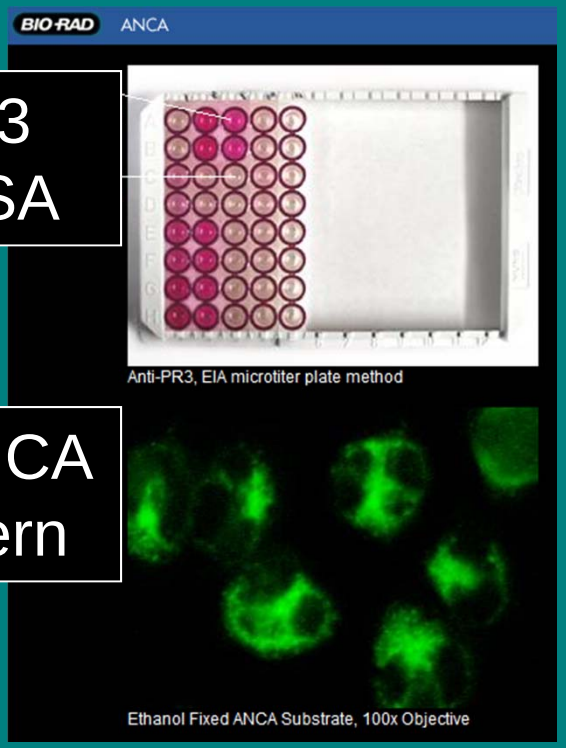
ELISAs For Distinguishing P-ANCA and C-ANCA

MPO ELISA



P-ANCA pattern

PR3 ELISA



C-ANCA pattern

- 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” Immunoasssays

“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

Indirect Immuno-fluorescence	Antigen (ELISA target)	Associated Disease(s)
ANCA	C-ANCA	Wegener's
	P-ANCA	Other Vasculitis, Inflammatory Bowel Disease
ANA	dsDNA	Systemic Lupus Erythematosis
	Ro/SSA	Systemic Lupus Erythematosis
	La/SSB	Sjogren's Syndrome
	Sm	Systemic Lupus Erythematosis
	Jo1	Polymyositis
	SCL-70	Scleroderma
AMA		Primary Biliary Cirrhosis
ASMA		Autoimmune Hepatitis
APCA		Pernicious Anemia
	TTG	Celiac Disease
	TPO	Thyroiditis (Graves and Hashimoto's)
	Tg	QA for use with Tg (recurrence of thyroid) cancer
	RF	Rheumatoid Arthritis
	CCP	Rheumatoid Arthritis

Self-Assessment Question 1

Which of the following combinations of autoantibody and disease is ***incorrect***?

A) ANA: Systemic Lupus Erythematosis
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