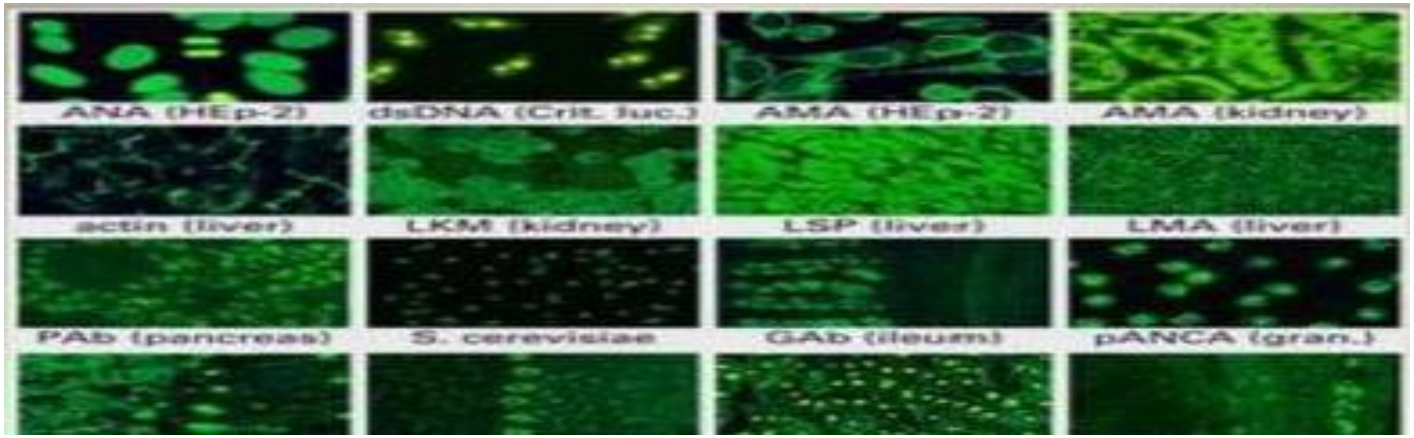

BASICS OF IMMUNOFLUORESENCE AND ITS APPLICATIONS IN MICROBIOLOGY

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IMMUNOFLUORESCENCE ASSAY

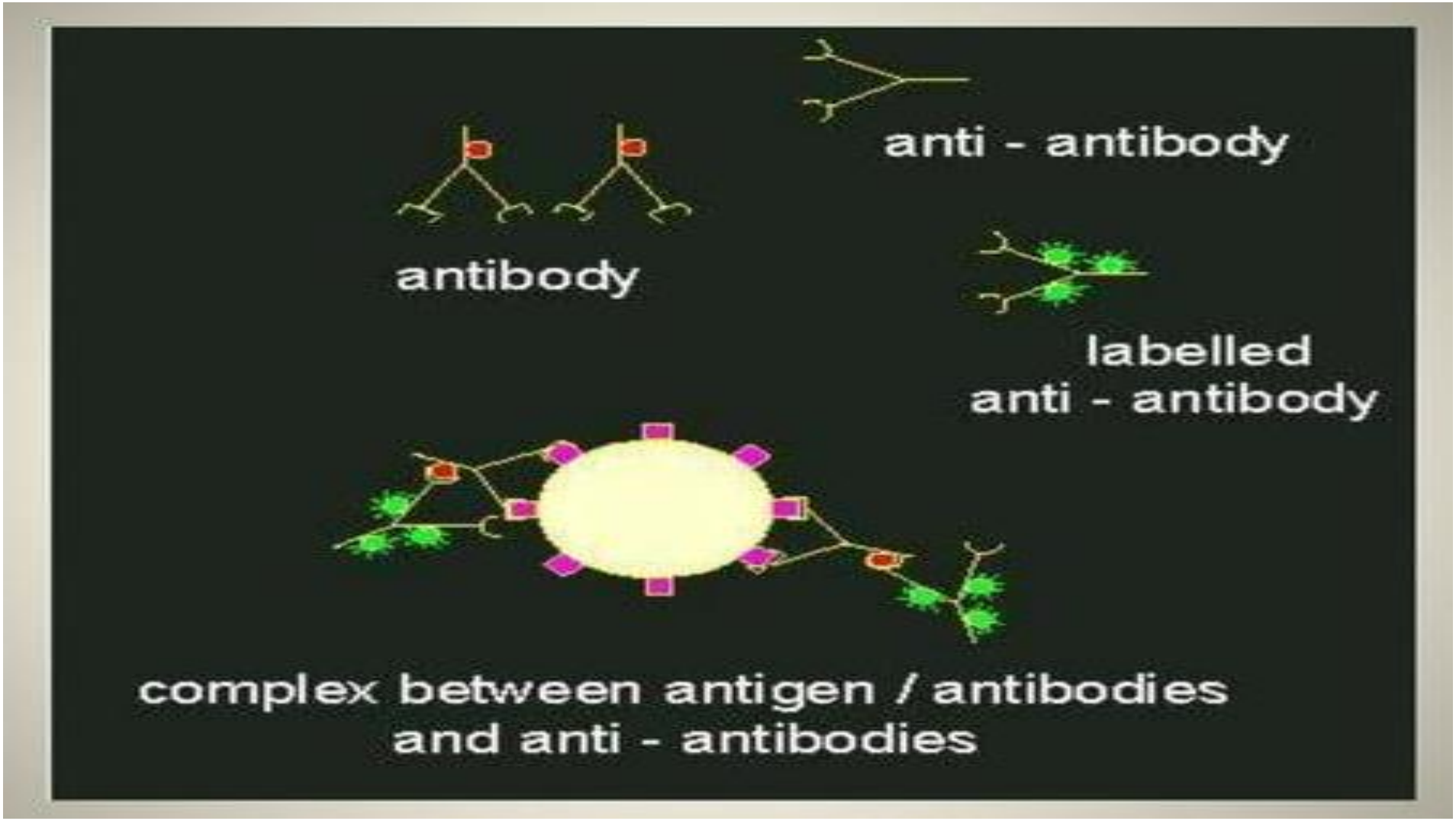
- Immunofluorescence is a technique allowing the visualization of a specific protein or antigen in tissue sections by binding a specific antibody chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC).
- The specific antibodies are labeled with a compound (FITC) that makes them glow an apple-green color when observed microscopically under ultraviolet light.

IMMUNOFLUORESCENCE ASSAY

- Fluorescence is the property of certain molecules or fluorophores to absorb light at one wave length and emit light at longer wave length (emission wavelength) when it is illuminated by light of a different wavelength (excitation wavelength).

- The incident light excites the molecule to a higher level of vibrational energy. As the molecules return to the ground state, the excited fluorophore emits a photon(= fluorescence emission).

PRINCIPLE OF THE TEST



IMMUNOFLUORESCENCE TYPES

- **1) direct immunofluorescence:** staining in which the primary antibody is labeled with fluorescence dye,
- **2) indirect immunofluorescence:** staining in which a secondary antibody labeled with fluorochrome is used to recognize a primary antibody.

DIRECT IMMUNOFLUORESCENCE TEST (DIF)

- DIF is a one-step procedure used to detect and localise immunoreactants deposited invivo in the patient's skin
- It permits early diagnosis, treatment and subsequent monitoring of disease activity
- The immunoreactants include antibodies, complement components and fibrinogen

DIF PROCEDURE

1
- Perilesional skin
- 3mm punch biopsy

2
- Transported in Normal saline
- Michel's media

3
- Snap frozen
- 4 -6microns thick slices are cut

4
- Incubated with IgG, IgA, IgM, C3

DIF PROCEDURE

5
- Incubated for 1hr

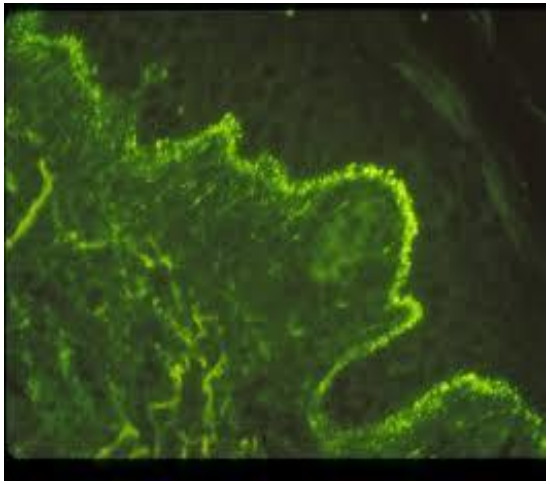
6
- Wash
- Mount with buffered glycerine

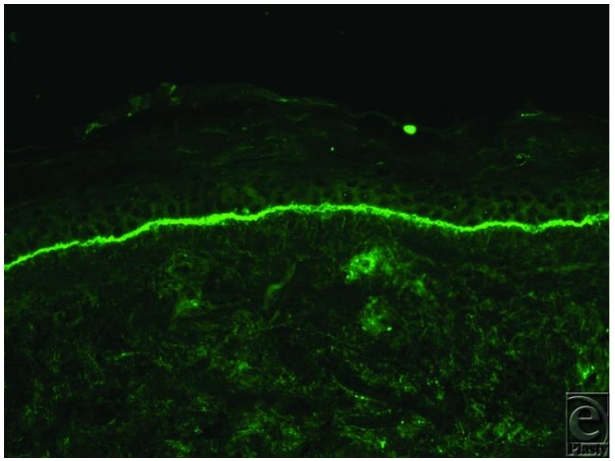
7
- Examined under fluorescence microscope

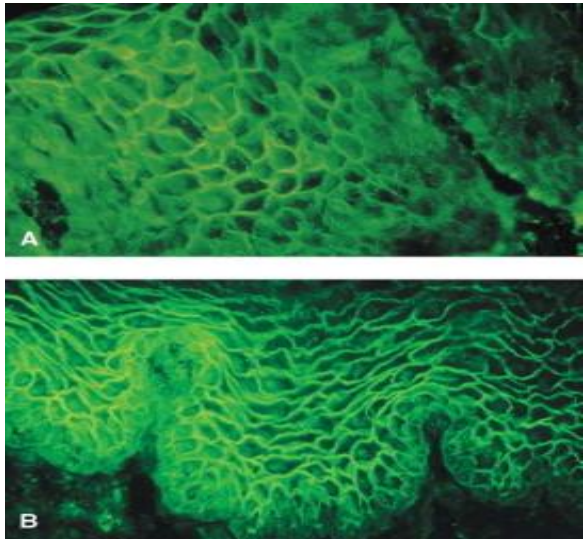
INTERPRETATION

- Nature of immune deposits: IgG, IgA, IgM, C3
- Site of immune deposits: Dermo-epidermal junction (DEJ), Intercellular spaces (ICS) in epidermis , blood vessels
- Semiquantitative grading of strength of
- fluorescence: + to ++++
- Pattern of immune complex deposits: granular , linear , lace- like

PATTERNS OF DEPOSITS







AgS INVOLVED IN PEMPHIGUS

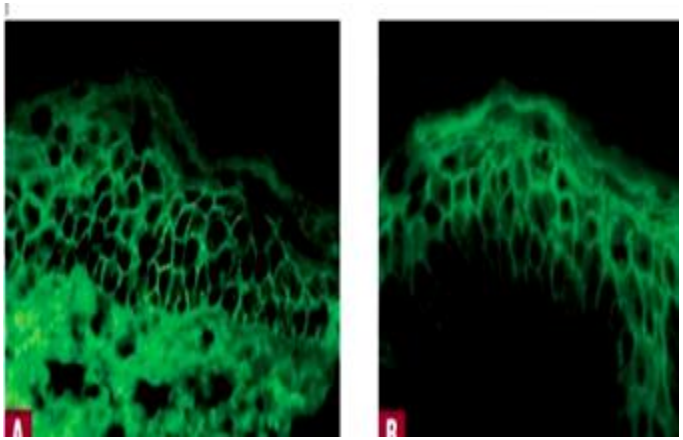
PEMPHIGUS TYPES	ANTIGENS TARGETED
PEMPHIGUS VULGARIS	DSG 3 & 1
PEMPHIGUS FOLIACEUS	DSG 1
Ig A PEMPHIGUS	DSG 1 & 3
PARANEOPLATIC PEMPHIGUS	DSG 1 & 3, DP

DSG- Desmogleins
DP- Desmoplakin

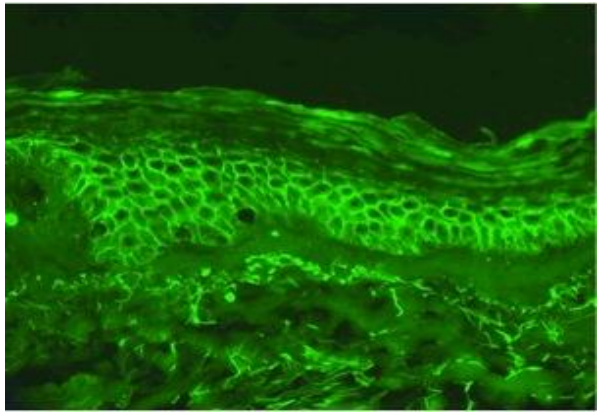
DIF IN PEMPHIGUS GROUP

PEMPHIGUS TYPES	SITE	PATTERN	IMMUNO-REACTANT
PEMPHIGUS VULGARIS	ICS	LACE -LIKE	Ig G
PEMPHIGUS FOLIACEUS	ICS	LACE -LIKE	Ig G
Ig A PEMPHIGUS	ICS	LACE -LIKE	Ig A
PARANEOPLATIC PEMPHIGUS	ICS	LACE -LIKE	Ig G
	BMZ	LINEAR	Ig G , C3
	BMZ	GRANULAR	Ig G, C3

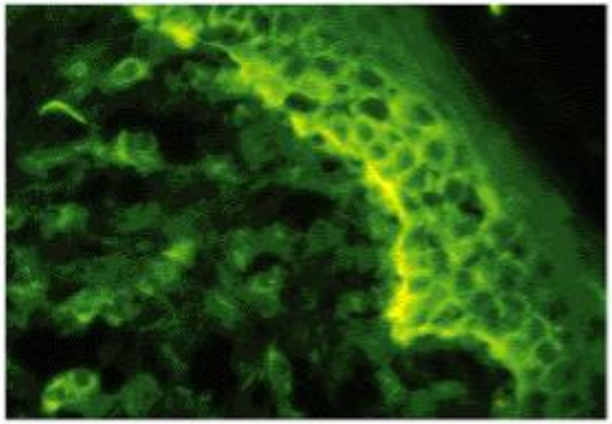
P.Vulgaris vs P.foliaceus



IgA PEMPHIGUS- DIF



PARANEOPLASTIC PEMPHIGUS

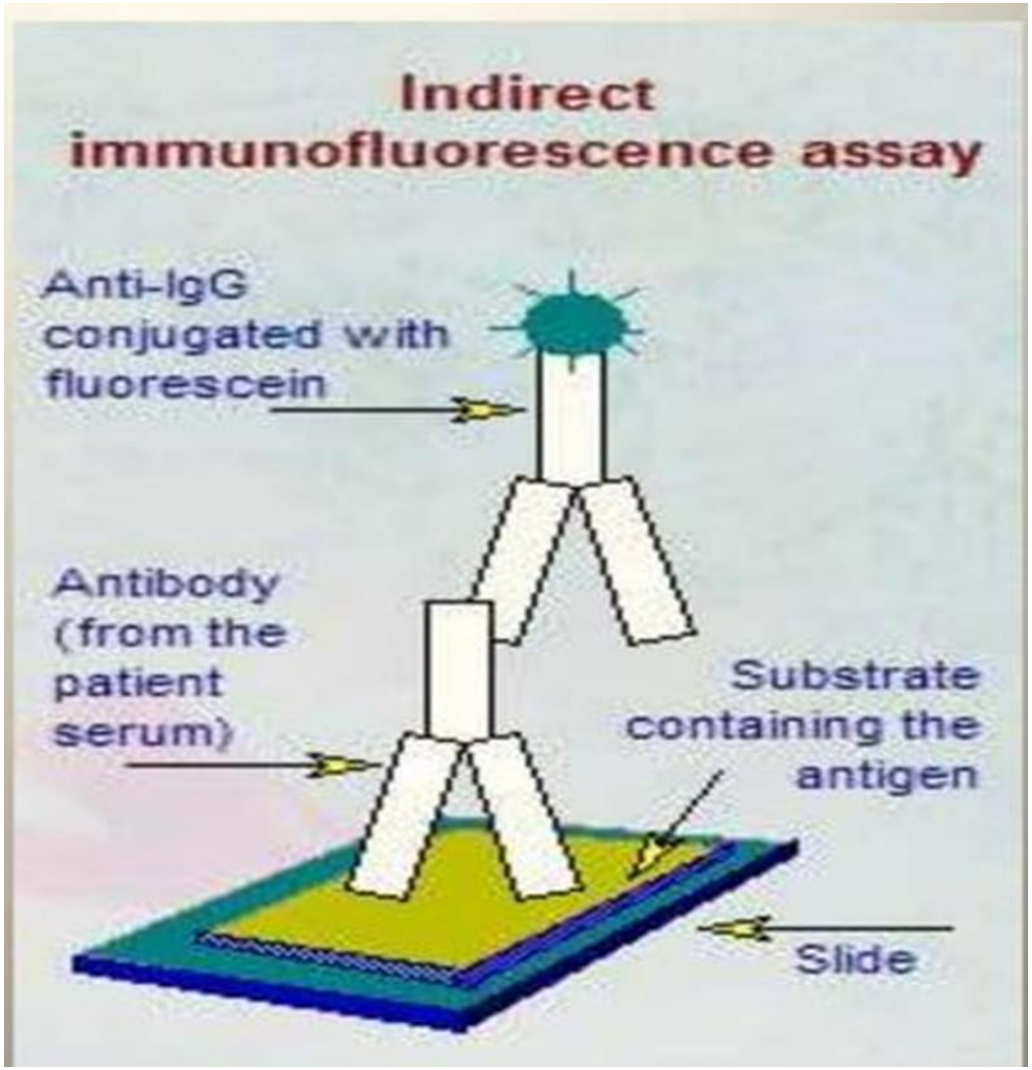


INDIRECT IMMUNOFLUORESCENCE

Indirect immunofluorescence uses two antibodies

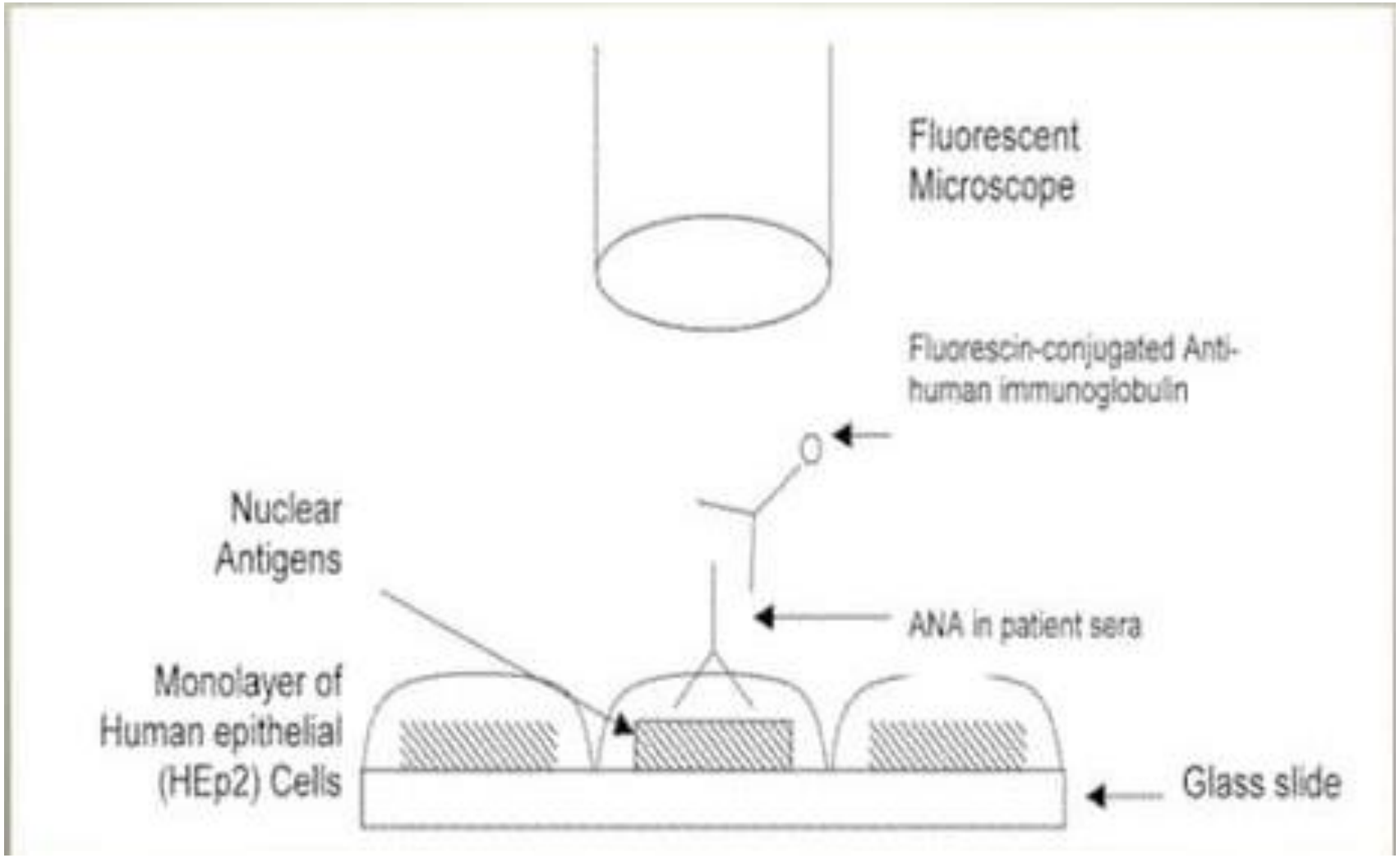
- the first (the primary antibody) recognises the target molecule and binds to it
- the second (the secondary antibody), which carries the fluorophore, recognises the primary antibody and binds to it.

PRINCIPLE OF IIF

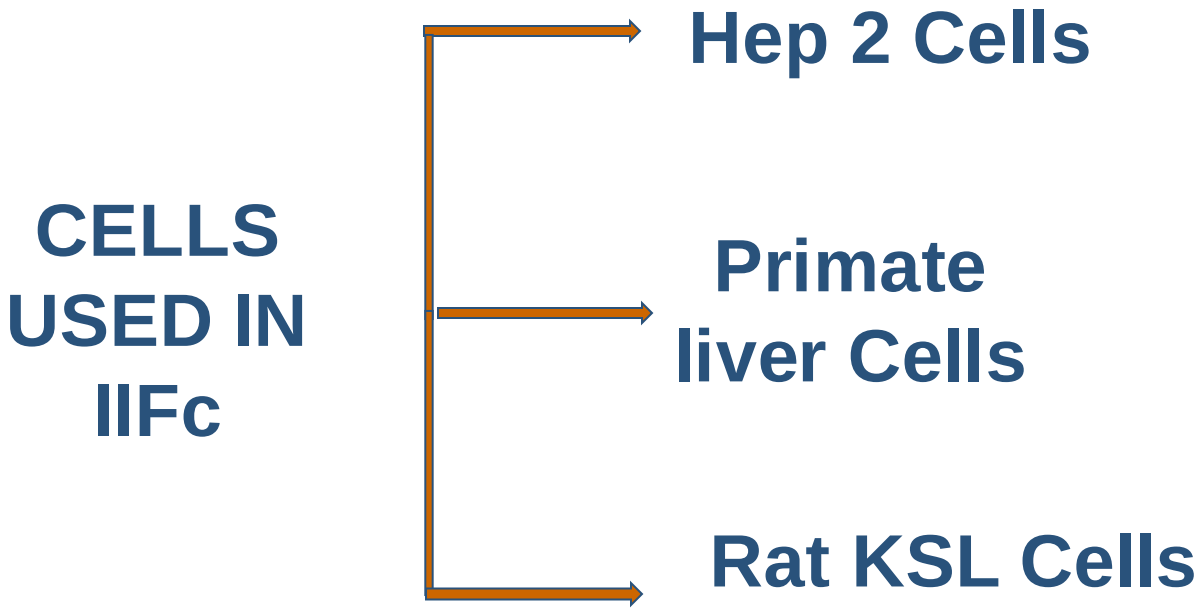


- For the determation of autoantibodies, tissue sections are used as antigen substrates.
- If the sample is positive, specific antibodies in the diluted serum sample attach to the antigens coupled to a solid phase.
- In a second step, the attached antibodies are stained with fluorescein-labelled anti-human antibodies and visualized with the fluorescence microscope.

PROCEDURE


Fluorescent
Microscope
Fluorescin-conjugated Anti-
human immunoglobulin
Nuclear
Antigens
ANA in patient sera
Monolayer of
Human epithelial
(HEp2) Cells
Glass slide

Substrates used In IIF



ANTIBODIES INVOLVED

Anti-Nuclear

1. ds DNA
2. Histone abs
3. Nucleosome
4. Nuclear membrane-lamins
5. Nuclear membrane – nuclear pores (gp120)

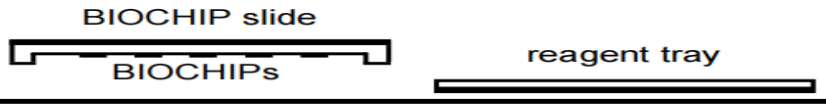
Anti-cytoplasmic

1. Jo-1 abs
2. PL7, PL12 abs
3. Rib-P abs
4. SRP abs

Antibodies seen with KSL tissues

1. AMA – m2 abs (anti-mitochondrial abs)
2. F-actin abs
3. LKM-1 abs
4. Gastric parietal cell abs

Procedure of IIF

TITERPLANE Technique		
Pipette:	30 µl per field	
Incubate:	30 min	
Wash:	1 s flush 5 min cuvette	
Pipette:	25 µl per field	
Incubate:	30 min	
Wash:	1 s flush 5 min cuvette	
Mount:	max. 10 µl per field	
Evaluate:	fluorescence microscopy	

ADVANTAGES OF IIF

- Gold standard for ANA screening
- Complete antigen spectrum (Cell Nuclei, Cytoplasm)
- One substrate (HEp-2010) - screening of 150 different Aab
- High Sensitivity by visual evaluation
- Screening by Elisa can give false positive if HEp cell extract is used.
- Screening by ELISA gives false negative results due to limited number of antigens.
- All the Ags are not detected by immunoblot.

Patterns seen with IIF

1) Nucleus Pattern

2) Cytoplasmic Pattern

3) Mitosis Pattern

Nucleus
Homogenous

Nuclear membrane

Nucleus granular

Nucleus dotted

Nucleus nucleolar

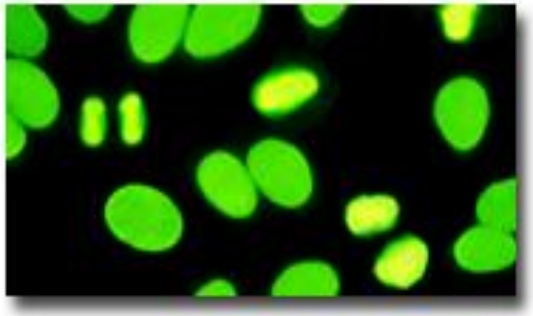
Cytoplasmic Granular

Cytoplasmic fine Granular

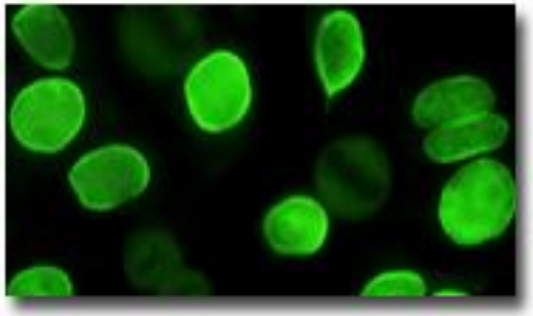
Cytoplasmic Filamentous

Nuclear patterns

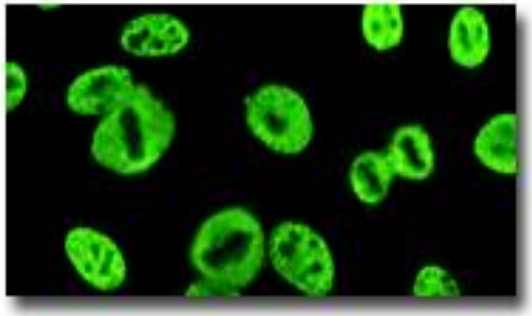
1. Nucl. homogeneous



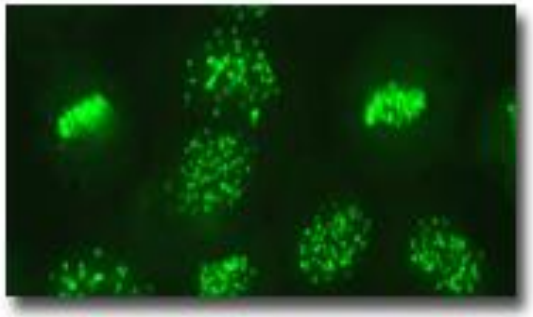
2. Nuclear membrane



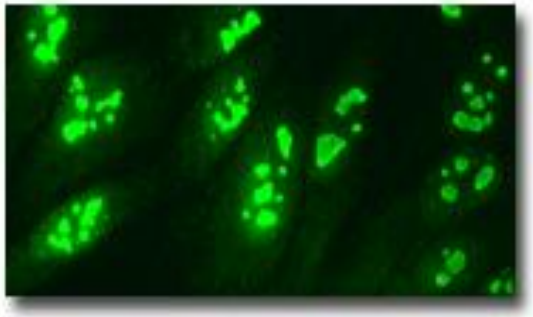
3. Nucleus granular



4. Nucleus dotted

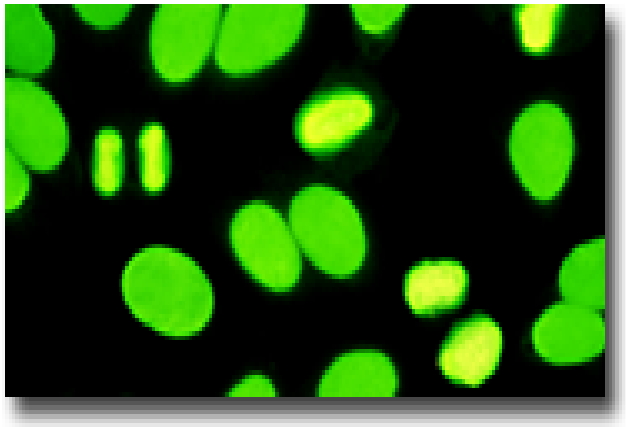


5. Nucleus nucleolare



Nuclear Homogeneous

Nuclear Homogeneous



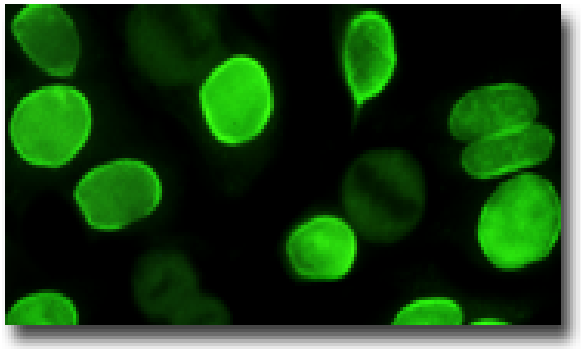
Autoantibodies against dsDNA

Autoantibodies against nucleosomes

Autoantibodies against histones

Nuclear Membranous

Nuclear membranous



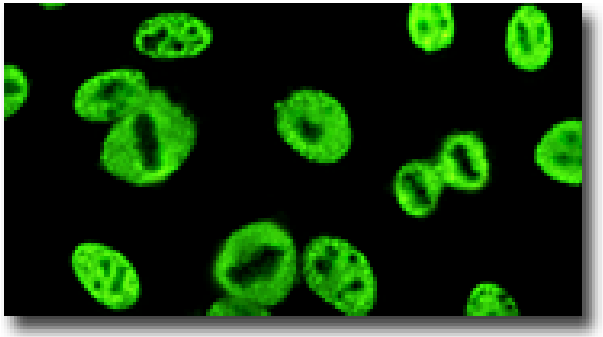
Autoantibodies against Lamin B receptor

Autoantibodies against Lamin A, B, C

Autoantibodies against gp210

Nuclear Speckled pattern

Nuclear Speckled pattern



Autoantibodies against nRNP/Sm

Autoantibodies against SS-A, SS-B

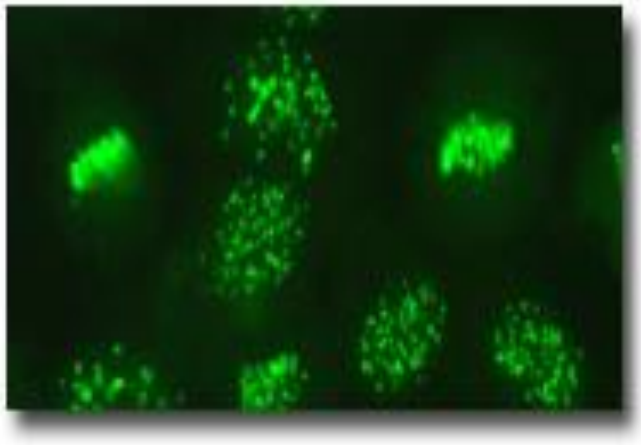
Autoantibodies against Ku

Autoantibodies against PCNA

Autoantibodies against Mitosin (CENP F)

Nuclear dotted pattern

Nuclear Dotted pattern



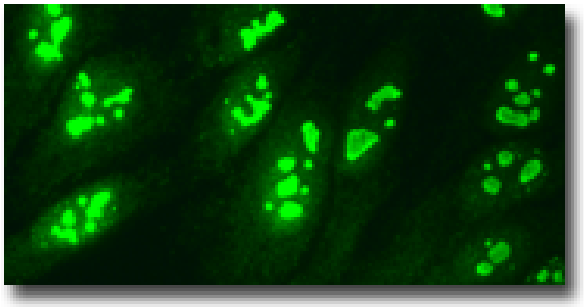
Nuclear dots

Few nuclear dots

Autoantibodies against Centromeres

Nucleolar pattern

Nucleolar Pattern



Autoantibodies against Scl-70

Autoantibodies against PM-Scl

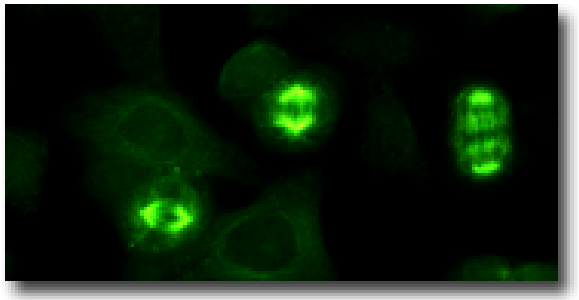
Autoantibodies against Fibrillarin

Autoantibodies against RNS-Polymerase I

Autoantibodies against NOR-90

Mitotic pattern

Mitotic pattern



Autoantibodies against spindle apparatus

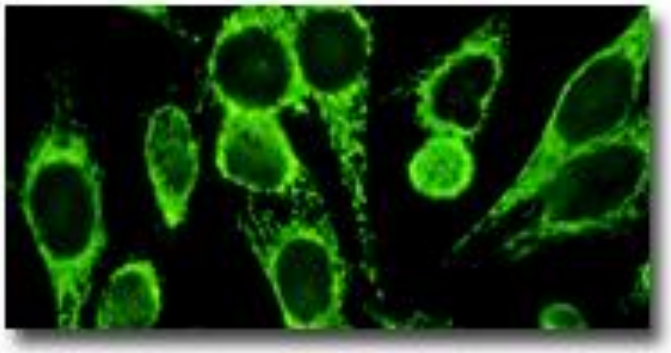
Autoantibodies against centrioles

Midbody

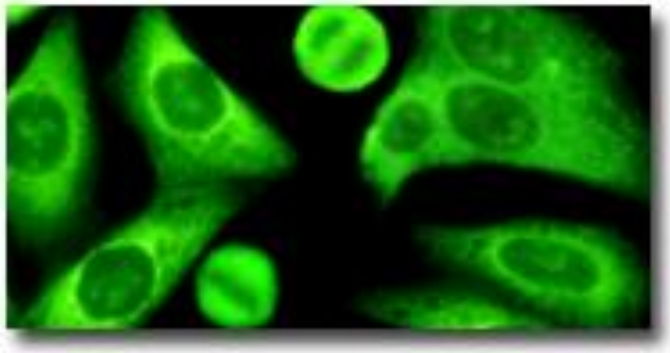
Antibodies against condensed chromosomes

Cytoplasmic patterns

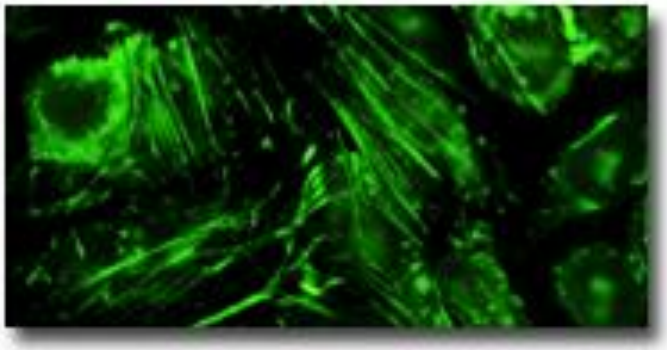
Cytoplasmic Granular



Cytoplasmic fine Granular

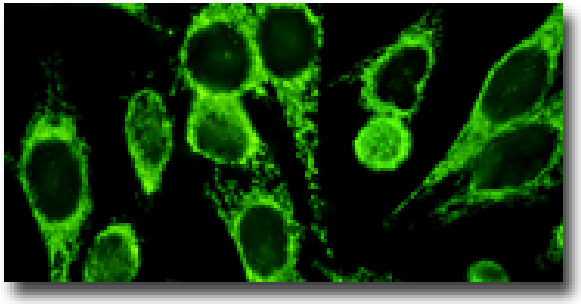


Cytoplasmic Filamentous



Cytoplasmic patterns

Cytoplasmic Granular Pattern



Autoantibodies against mitochondria (AMA)

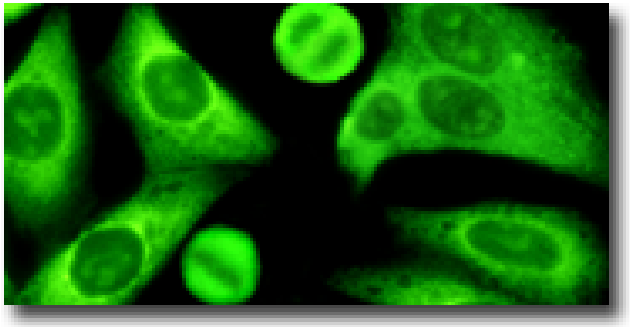
Autoantibodies against Jo-1

Autoantibodies against lysosomes

Autoantibodies against golgi apparatus

Cytoplasmic patterns

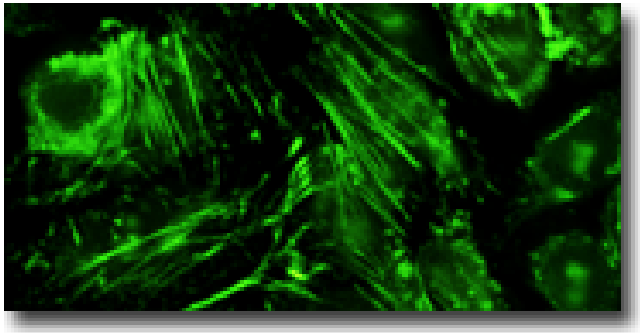
Cytoplasmic Fine Granular Pattern



Autoantibodies against rib. P-Protein

Cytoplasmic patterns

Cytoplasmic Filamentous Pattern



Autoantibodies against Actin

Autoantibodies against Vimentin

Autoantibodies against Tropomyosin

ANCA

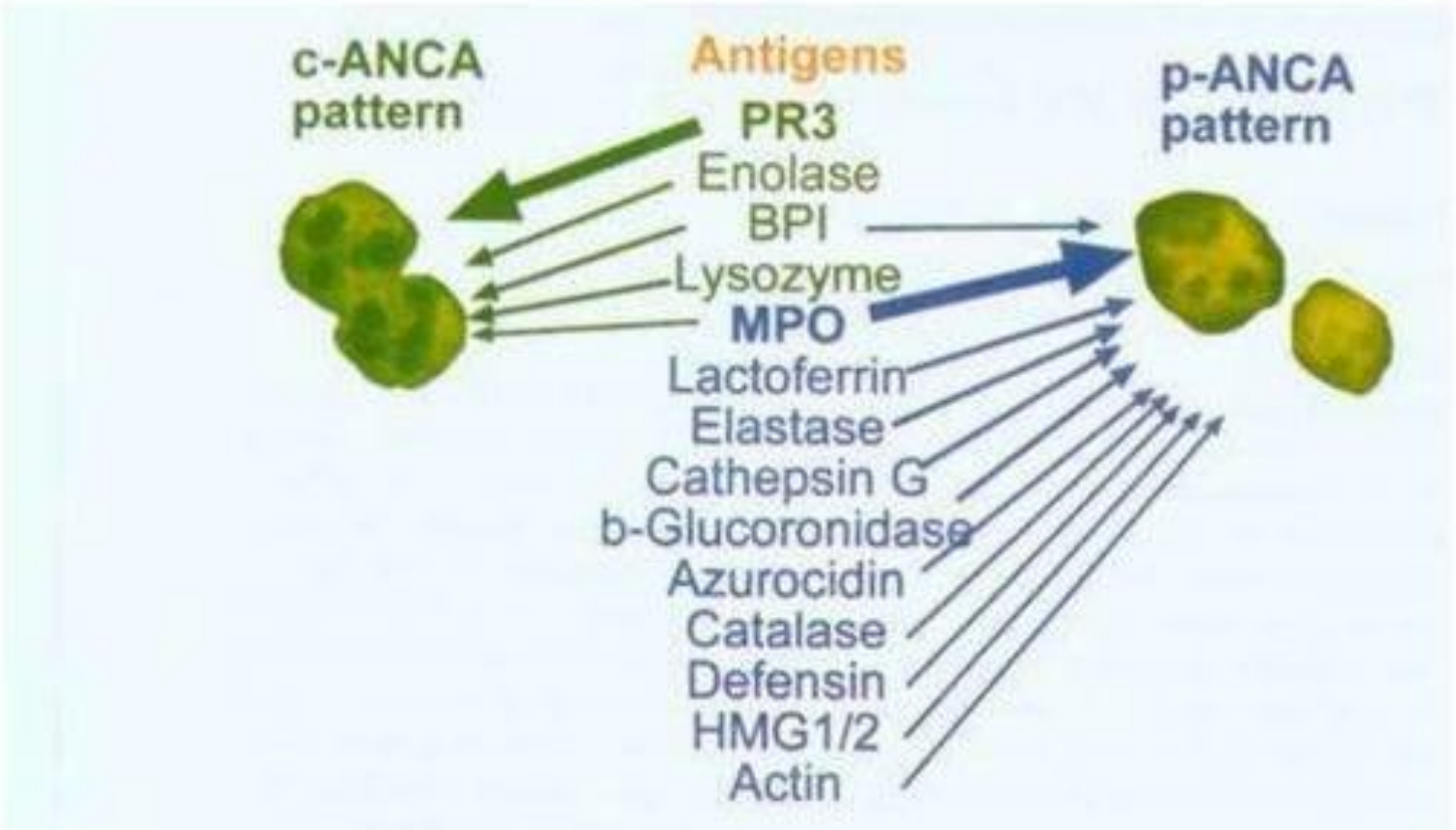
- ANCA auto antibodies related to inflammatory disorders.

- van der Woude et *al.* in **1985** showed ANCA related to **Wegener's granulomatosis**.

Ags involved

- Two main enzymes in ANCA take part in the killing of bacteria by:
  - *Proteinase 3 (PR3) showing cANCA pattern.*
  - *Myeloperoxidase (MPO) showing pANCA pattern.*

Other Antigens



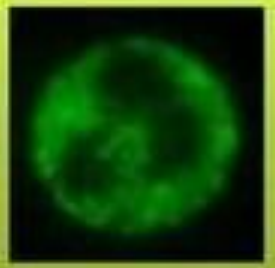
Development of ANCA

- *Theory of molecular mimicry.*
  - Superantigens have the power to stimulate a strong immune response . THEY have regions that resemble self-antigens – this is the theory of molecular mimicry.
  - classical example in post group A streptococcal rheumatic heart disease, where there is similarity between M proteins of *Streptococcus pyogenes* to cardiac myosin and laminin.
- *Theory of defective apoptosis.*
  - ANCA may be developed either via ineffective apoptosis or ineffective removal of apoptotic cell fragments, leading to the exposure of the immune system to molecules normally sequestered inside the cells. This theory solves the paradox of how it could be possible for antibodies to be raised against the intracellular antigenic targets of ANCA.[4]

ANCA patterns

p-ANCA, show a *perinuclear staining* pattern

c-ANCAs, show a *diffusely granular, cytoplasmic staining* pattern



Atypical that develop against *antigens other than MPO or PR3* will occasionally result in *patchy staining*

Clinical Significance of ANCA

C – ANCA: Cytoplasmic ANCA

- Abs against proteinase-3 (PR-3) seen in Granulomatosis with polyangitis (Wegener‘s Granulomatosis)

P – ANCA: Perinuclear ANCA

- Abs against Myeloperoxidase (MPO) seen in

a) Microscopic polyangitis (MPA)

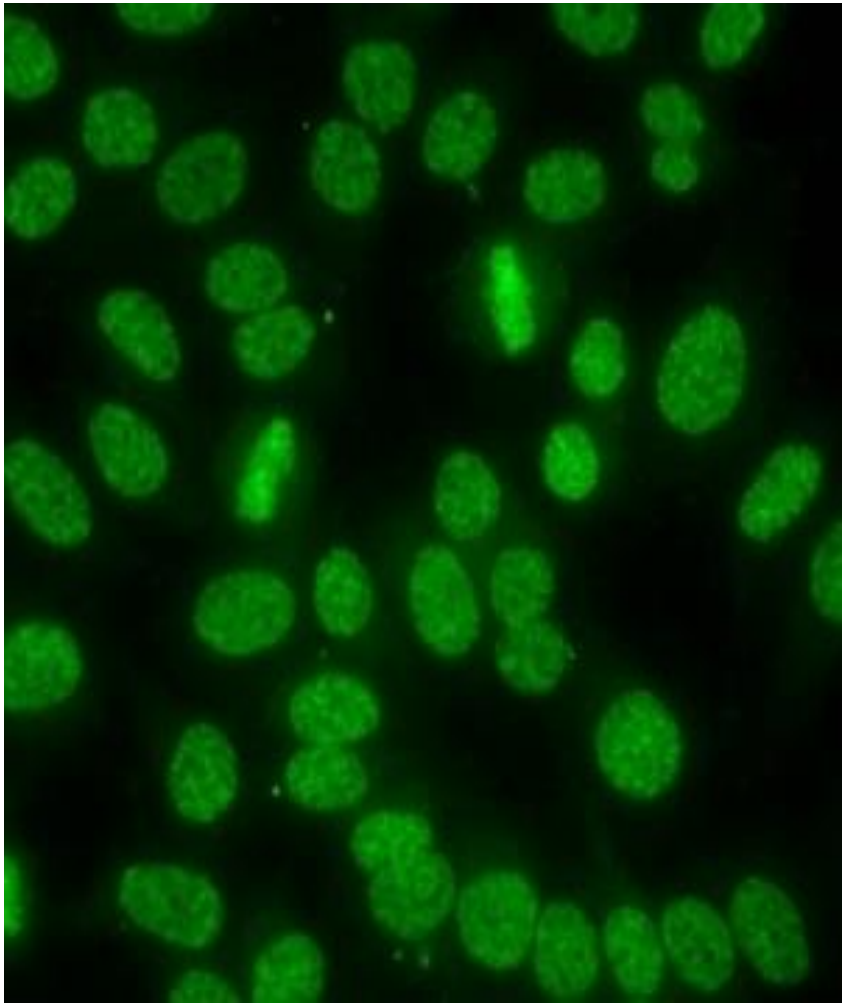
b) Eosinophilic Granulomatosis with polyangitis (EGPA) also known as Chrug-Strauss Syndrome.

DFS Pattern

Speckled pattern distributed throughout the interphase nucleus with characteristic heterogeneity in the size, brightness and distribution of the speckles. Throughout the interphase nucleus, there are some denser and looser areas of speckles (very characteristic feature).

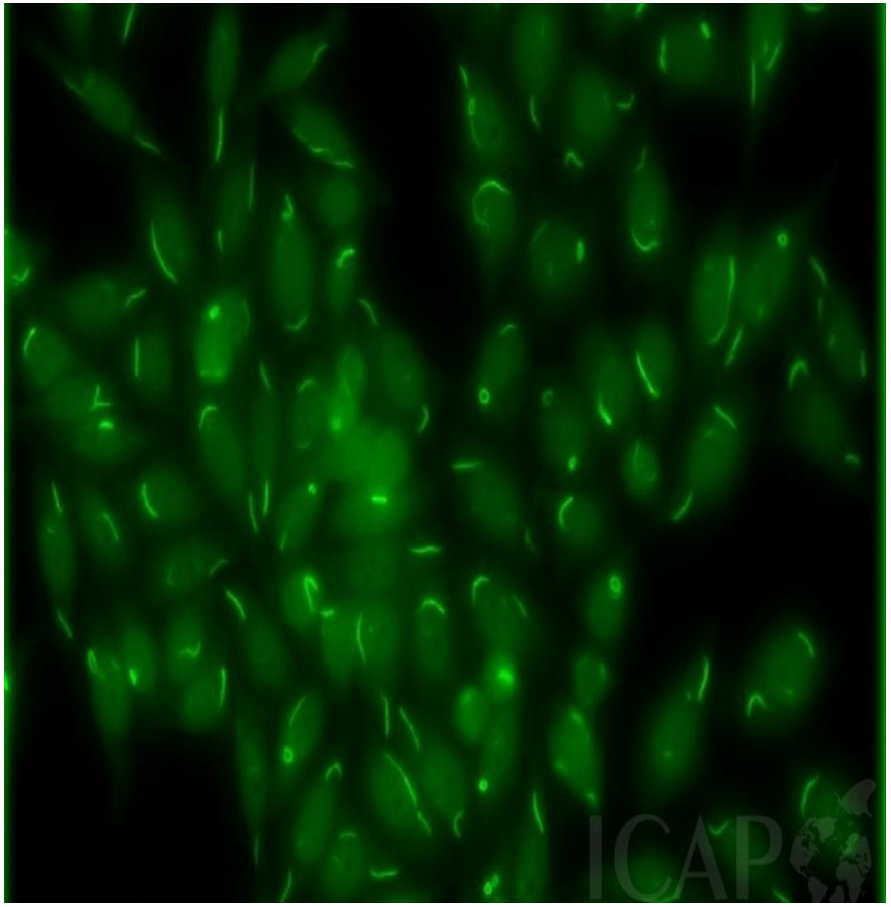
The metaphase plate depicts strong speckled pattern with some coarse speckles standing out.

Clinical Association: Both in apparently healthy individuals as well as patients who do not have a SARD.



Rings and Rods

Rings and Rods

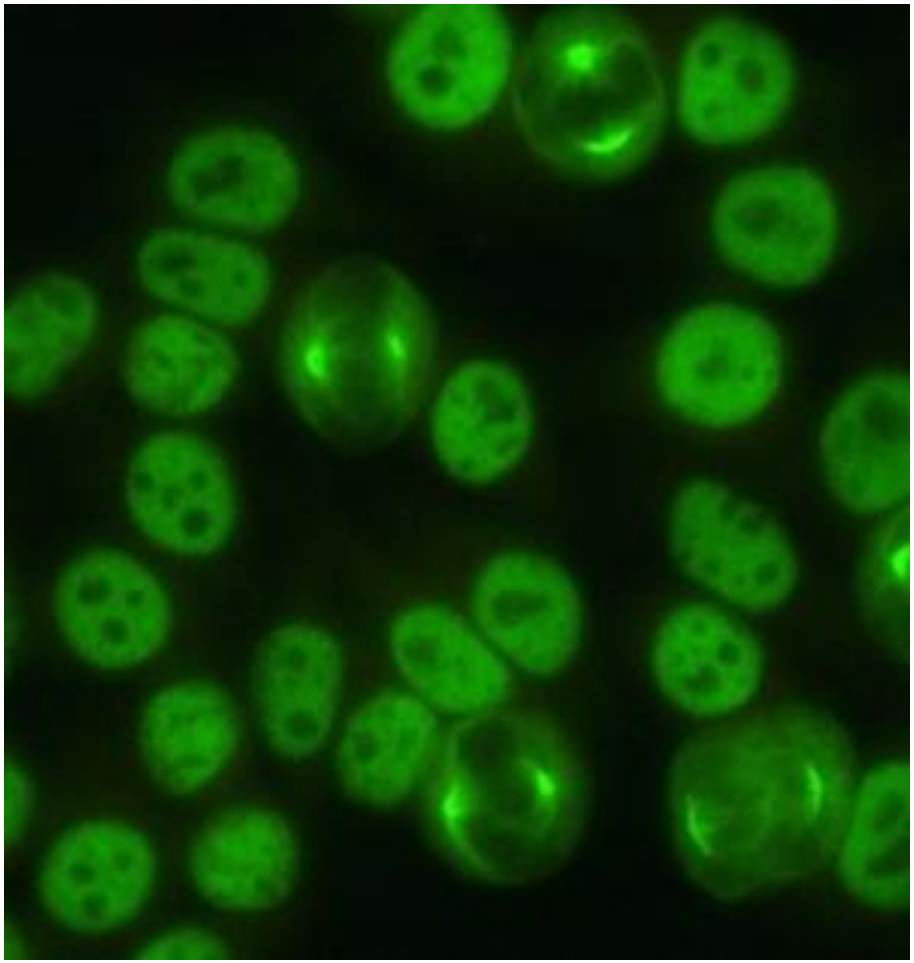


Most commonly found in HCV patients who have been treated with pegylated interferon - **α/ribavirin combination therapy**

NuMa

NuMa

- Nuclear speckled staining with spindle fibers.
- Approximately one-half of the patients with the AC-26 pattern have clinical features of a SARD (SjS, SLE, UCTD, limited SSc, or RA)



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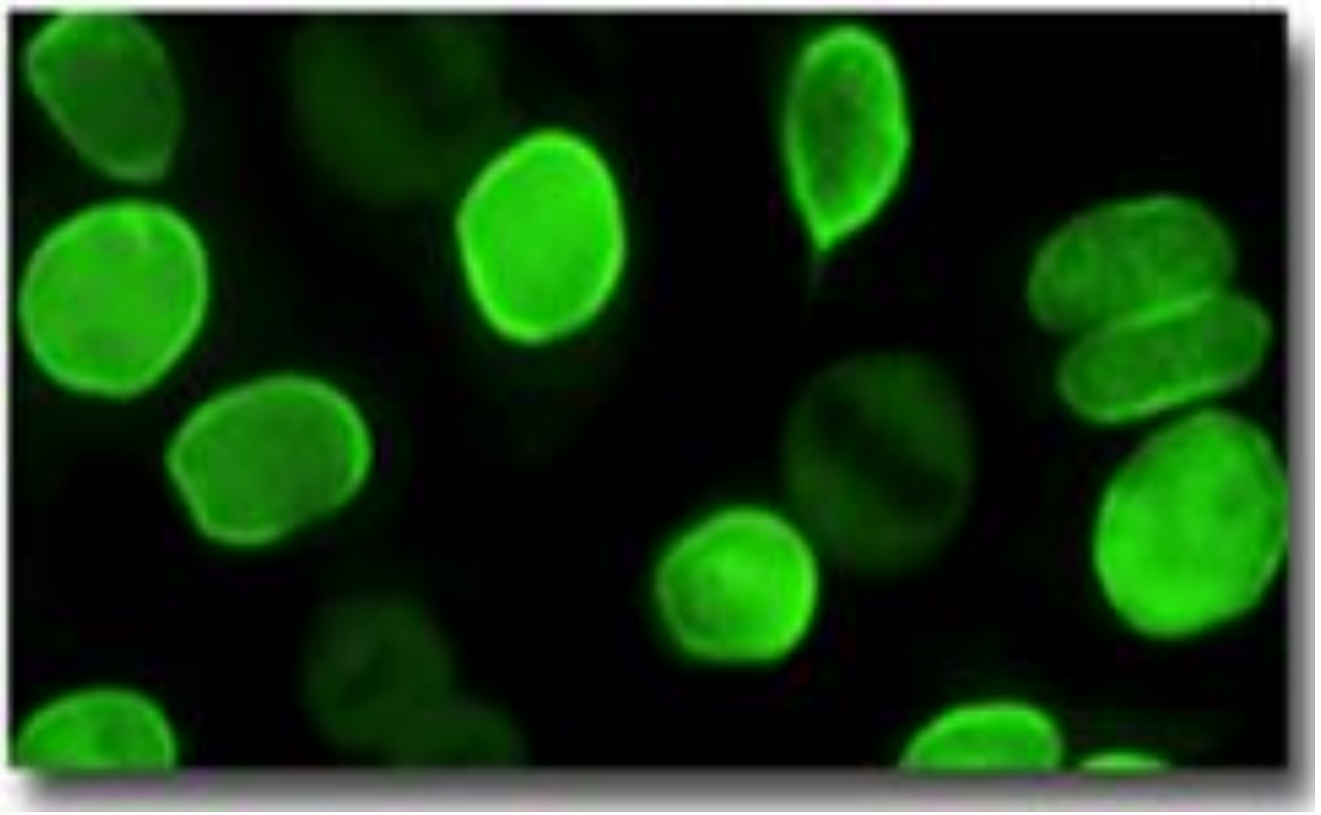

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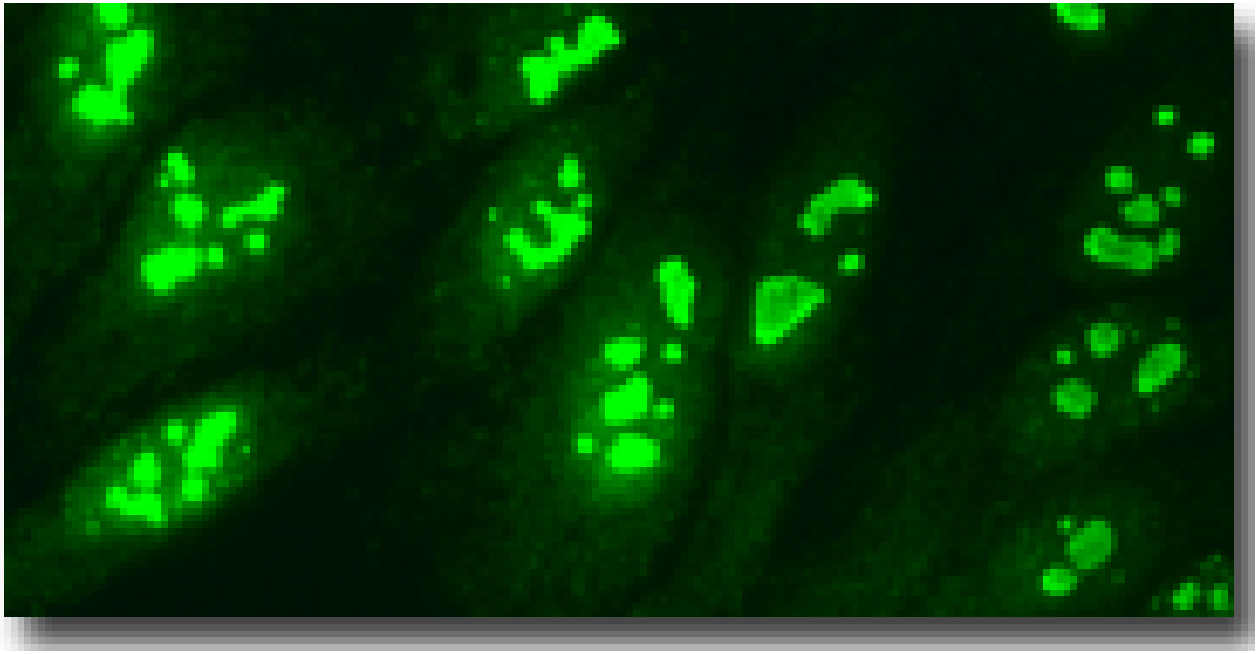

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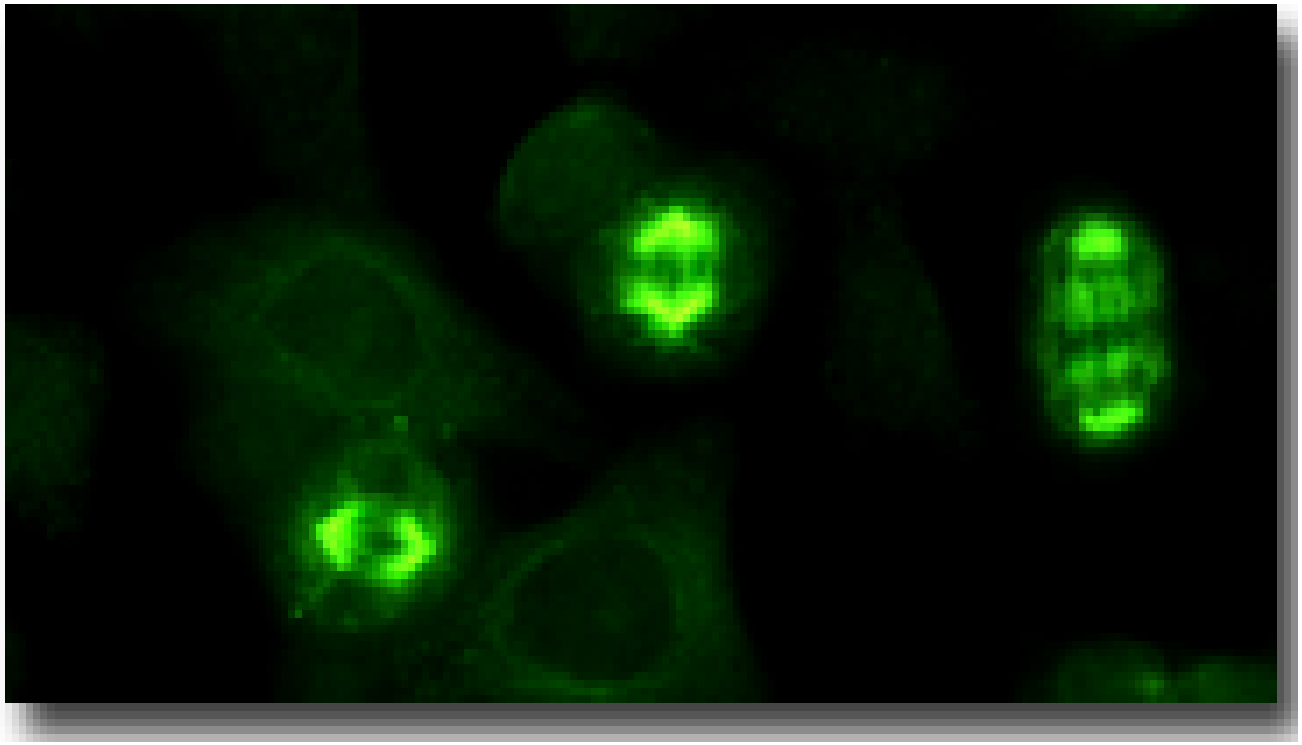

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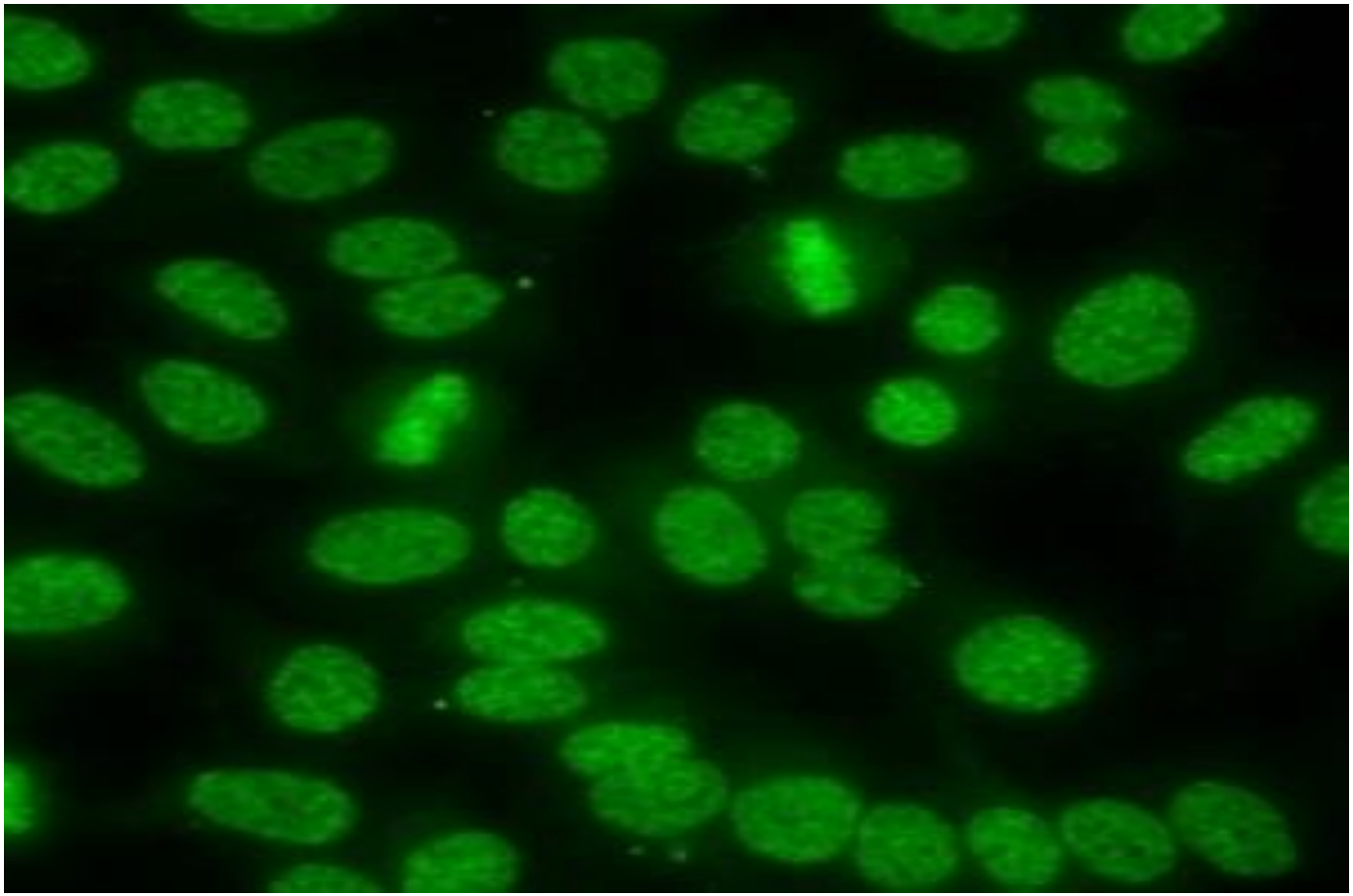

References

- ImmunoFluorescence Guide for the modern autoimmunity laboratory, 2013. Barbara Fabian.
- Kuby Immunology, 9th edition.
- Euroimmune immunoFluorescence guide, ANAIFA
- Seema Chabra *et a*l., PGIMER, 2012, Immunofluorescence in Dermatology.
- Pollock et al, 2002, *Journal of Cllinical Pathology*, Immunofluorescence patterns produced by ANCA vary depending on neutrophil substrate and conjugate,

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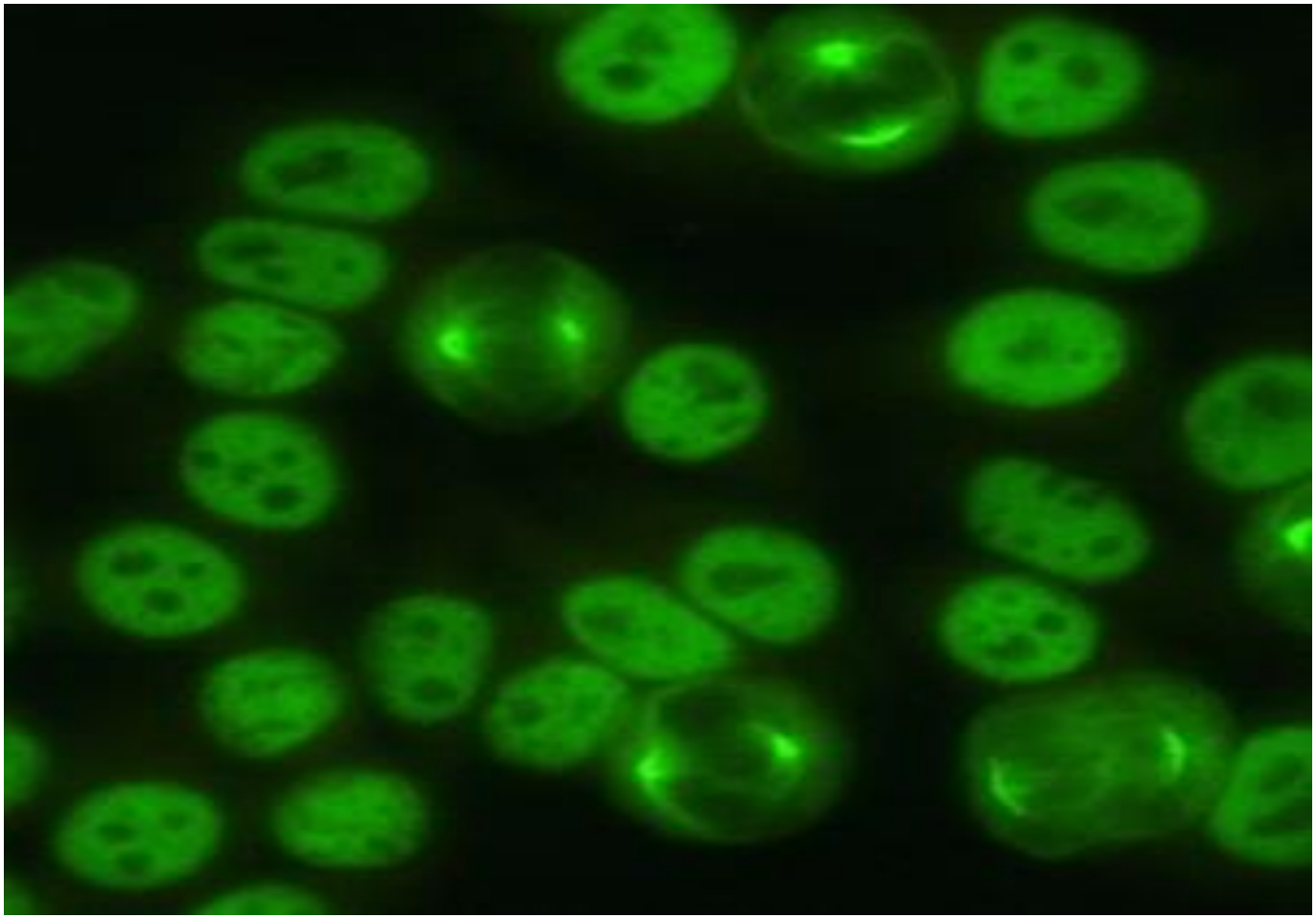

QUESTIONS???

THANK YOU