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Discovery

Screening of auto antibodies using indirect immunofluorescence in autoimmune predisposed individuals

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General Note
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ABSTRACT

Autoimmune diseases arise from an inappropriate immune response of the body to its own cell components. The body loses its ability to distinguish between self and non-self thereby auto-antibodies against own cellular components are produced. These auto-

antibodies were screened using Antinuclear antibody test (ANA) and characteristic patterns so formed were visualized using Immuno Fluorescent microscopy (IF). Screening and detection of auto-antibodies plays an important role in the diagnosis of various connective tissue disorders (CTD) and help to serve as important serological markers. The present study focuses on identifying and characterizing cell nuclear and cytoplasmic auto-antibodies using HEp-20-10 and primate liver as substrates in autoimmune predisposed individuals. 50 serum samples from patients with clinical symptoms were collected and analyzed for the presence for auto antibodies by IF microscopy. 30 samples showed positive results for auto antibodies. The antinuclear antibody indirect immuno fluorescence patterns were projected to visualize a certain spectrum of specific antibodies such as cytoplasmic granular (JO-1, 37%), homogeneous (23%) and nucleoplasm fine granular (13%), nucleoplasm coarse granular (7%) mixed pattern (7%), cytoplasm positive (7%), nucleoli fine and coarse granular (3%) each.

Keywords: Immunofluorescence microscopy; Anti-nuclear antibodies; Immunoblotting; Connective tissue disorders; Biochip; Titer plane.

Abbreviations: ANA - Anti nuclear antibody; PBS-Phosphate Buffer Saline; SLE – Systemic Lupus Erythromatosis; MCTD - Mixed connective Tissue; PCNA - Proliferating cell nuclear antigen; IIF - Indirect Immuno Fluorescence; CTD - Connective tissue disorder.

1. INTRODUCTION

Antibodies develop in our immune system to help the body fight infectious organisms. When an antibody recognizes the foreign proteins of an infectious organism, it recruits other proteins and cells to fight off the infection. This cascade of attack is called inflammation. Unfortunately, some antibodies make incorrect calls, identifying a naturally-occurring protein (or self-protein) as foreign. These auto-antibodies start the cascade of inflammation, causing the body to attack itself. Any disease that results from such an aberrant immune response is termed as autoimmune disease (Davidson et al., 2007). A characteristic feature of many systemic autoimmune diseases is the presence of circulating antibodies directed against cellular constituents called antinuclear antibodies (ANA's). Indirect immunofluroscence can be considered as a powerful, sensitive and comprehensive test for screening auto-antibodies. Tissue culture cells lines, such as the human laryngeal epithelial cancer cell line (HEp-20-10 cells), are used most commonly in IIF testing. Such cell lines are more efficient substrates, have large nuclei and nucleoli, have a higher frequency of certain antigens, and are less costly (Fritzler, 2006). There are six known main patterns of fluorescence that indicate different autoantibody specificities: homogeneous nuclear pattern, peripheral nuclear pattern, speckled nuclear pattern, nucleolar pattern, centromere pattern, and cytoplasmic pattern (Bradwell et al., 1999).

2. MATERIALS AND METHODS

2.1. Sample Collection and Processing

Blood samples were collected from 50 patients with clinical symptoms. These samples were centrifuged and serum was extracted. Serum was then processed. The sample to be investigated was diluted 1:10 in PBS-Tween. 10.1µl of sample was diluted in 100µl of PBS-Tween and mixed thoroughly.

2.2. Titer plane Technique

Samples were applied to the titer plane. The BIOCHIP Slides were then placed into the recesses of the titer plane, where all BIOCHIP's of the slides come into contact with the samples, and the individual reactions commence simultaneously. As the serum was confined to a closed space, there was no need to use a conventional "humidity chamber". Using titerplane, it was possible to incubate any number of samples next to each other and simultaneously under identical conditions (Fig.1).

2.3. Methodology

25µl of diluted sample was applied to each reaction field of the reagent tray without any air bubbles. Then the Biochip slide was fitted into the corresponding recesses of the titer plane. The samples were incubated at room temperature for 30 minutes. Then the Biochips were rinsed with a flush of PBS-tween using a beaker and then it was immersed in a coupling jar containing PBS-tween for 5 minutes. 20µl of fluorescein-labeled anti-human globulin was added to each reaction field of a clean titer plane. Then Biochip slide was removed from the coupling jar and within five seconds the back and the long sides of the Biochip slide was blotted with a tissue paper. The Biochip slide was immediately put into the recesses of the titer plane. Again the biochip was incubated at room temperature for 30 minutes. The Biochip slides were rinsed again with a flush of PBS-tween using a beaker and then it was immersed in a coupling jar containing PBS-tween for 5 minutes. 10µl of glycerol was placed onto a cover glass. The biochip was removed from

the coupling jar and it was dried. The biochip facing downwards was put onto the cover glass. The fluorescence was read with the microscope initially in objective 20X and then focused using 40X (Fig.2)

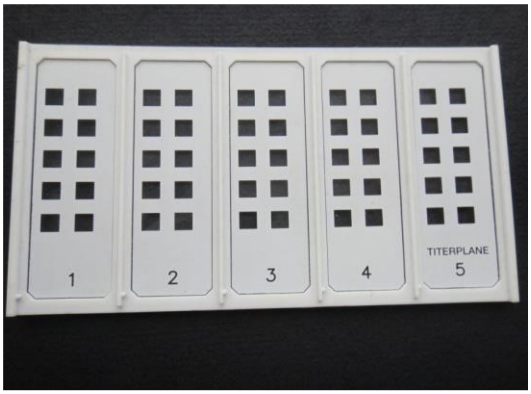

Figure 1
TiterPlane

Figure 2
Indirect Immunofluorescence Microscope

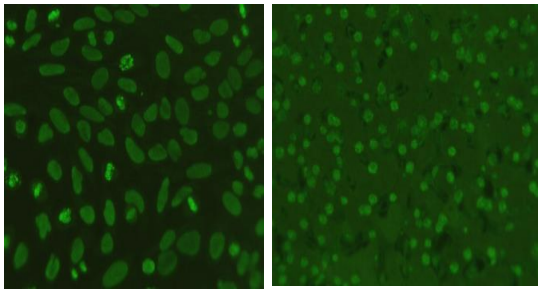

Figure 3
Positive Control

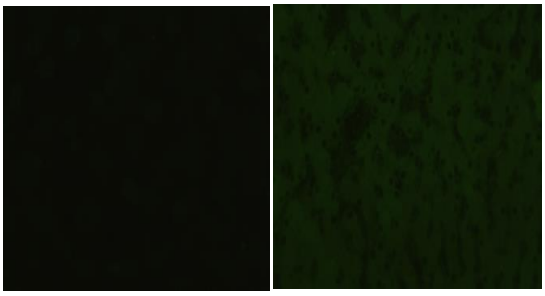

Figure 4
Negative Control

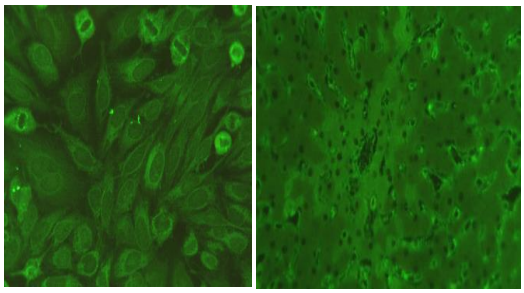

Figure 5
Cytoplasmic pattern

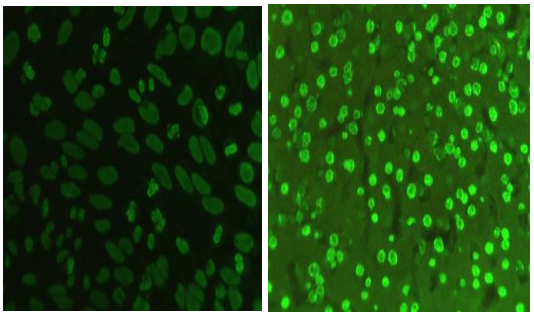

Figure 6
Homogenous Pattern

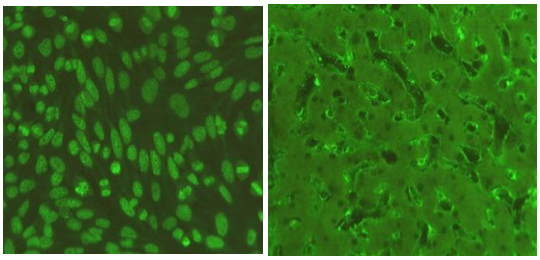

Figure 7
Nucleoplasm fine granular

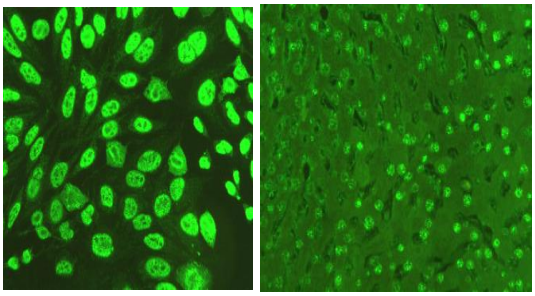

Figure 8
Nucleoplasm coarse granular

3. RESULTS AND DISCUSSION

3.1. Screening for Auto antibodies by IIF

One of the hallmarks of many autoimmune diseases is the production of auto-antibodies to specific cascades of cellular antigens. ANA against variety of nuclear antigens can cause a positive ANA-IIF. IIF pattern strongly suggest a distinct specificities against specific nuclear and cytoplasmic antigens. Additional test are done to confirm the presence of ANA against specific type of antigens through ELISA or Immunoblotting. Auto-antibodies together with other risk factors are very helpful in early therapy decisions. Early therapy may possibly prevent or delay severe complication

In our present study, 50 serum samples which satisfied the definite selection criteria are processed for ANA test. Thirty (60%) of serum samples were positive where in the fluorescent ANA pattern could be correlated with the presence of specific antibodies. Cytoplasmic granular, the most common ANA pattern observed in this study (n=11, 36.38%) shows an association with JO-1. In this fine granules were observed in the cytoplasmic region and the nuclear region was negative. The next common pattern is cell nuclei homogeneous (n=7, 23%) showed an association with dsDNA, nucleosomes and histones. In this uniform fluorescence was observed. Nucleoplasm fine granular pattern were seen in four cases (13%). These are associated with SSA, SSB and in these fine granules were observed and the cytoplasmic area is negative. Nucleoplasm coarse granular and cytoplasmic positive patterns were seen only in 2 cases each (6.5%). nRNA/Sm auto-antibodies were seen in nucleoplasm coarse granular pattern whereas Ribosomal P Proteins was seen in cytoplasmic positive patterns. In nucleoplasm coarse granular pattern coarse granules were observed and in the case of cytoplasmic positive homogenous to fine granules were observed inside the cytoplasm. One nucleoli fine granular and nucleoli coarse granular patterns (3.25%) each were seen which is associated with Fibrillarin and RNA Polymerase-1 respectively. In the case of nucleoli fine granular pattern, fine droplets were observed and in the case of nucleoli coarse granular pattern chromosomal area was negative (Table 1 and Fig.3 to Fig.14).

In two cases (6.5%) mixed pattern were observed, sample 15 showed cytoplasmic positive with nucleoplasm granular pattern which correlates with SLE and sample 40 showed Cytoplasm coarse granular and nuclear dots which strongly correlates with Primary biliary cirrhosis.

Table 1 Representing the pattern identified with the associated auto antibodies and diseases

S.No	Sample ID	Pattern Identified	Auto antibodies	Disease Associated
1	2	Cytoplasmic granular	JO-1	Polymyositis
2	3	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
3	5	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
4	6	Cytoplasmic granular	JO-1	Polymyositis
5	7	Cytoplasmic granular	JO-1	Polymyositis
6	9	Cytoplasmic granular	JO-1	Polymyositis
7	10	Nucleoplasm fine granular	SS-A/SS-B	SLE, Sjögren's syndrome
8	13	Nucleoplasm coarse granular	nRNA/Sm	SLE, MCTD
9	15	Mixed pattern (cytoplasmic positive + nucleoplasm)	SS-A/SS-B, AMA	SLE
10	16	Nucleoli coarse granular	RNA Polymerase1	Progressive systemic sclerosis
11	18	Cytoplasmic granular	JO-1	Polymyositis
12	20	Nucleoplasm fine granular	SS-A/SS-B	SLE,Sjögren's syndrome
13	21	Nucleoplasm fine granular	SS-A/SS-B	SLE, Sjögren's syndrome
14	22	Cytoplasmic positive	Ribosomal P protein	SLE
15	24	Cytoplasmic granular	JO-1	Polymyositis
16	26	Cytoplasmic granular	JO-1	Polymyositis
17	28	Nucleoli fine granular	Fibrillarin	Progressive systemic sclerosis
18	31	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
19	33	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
20	36	Nucleoplasm fine granular	SS-A/SS-B	SLE,Sjögren's syndrome
21	37	Cytoplasmic granular	JO-1	Polymyositis
22	40	Mixed pattern (cytoplasm coarse granular +	AMA, Nuclear dots	Primary biliary cirrhosis

		nuclear dots)		
23	41	Cytoplasmic positive	Ribosomal P Protein	SLE
24	42	Cytoplasmic granular	JO-1	Polymyositis
25	43	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
26	45	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
27	46	Nucleoplasm coarse granular	nRNA/Sm	SLE, MCTD
28	47	Cell nuclei homogenous	dsDNA, nucleosome, histones	SLE, Drug induced lupus, MCTD
29	49	Cytoplasmic granular	JO-1	Polymyositis
30	50	Cytoplasmic granular	JO-1	Polymyositis

Our present study showed JO-1 as the predominant antibody (36.38%), marker for people with Polymyositis (PM). In disease condition many muscles get inflamed. Individuals with JO-1 antibodies often have a distinct syndrome called "Antisynthetase syndrome". Symptoms include pain, with marked weakness and/or loss of muscle mass in the proximal musculature, particularly in the shoulder and pelvic girdle. Polymyositis is found mostly in people over the age of 20 and affects more women than men.

The disease associated with nucleus homogenous pattern is SLE, Rheumatoid arthritis. SLE may affect the skin, joints, kidneys, brain, and other organs. It may occur at any age, but appears most often in people between the ages of 10 and 50 and its more common in women. In Rheumatoid Arthritis, immune system attacks the body's own tissues, specifically the synovium, and a thin membrane that lines the joints. This causes pain in the joints and inflammation that is systemic. Rheumatoid arthritis most commonly begins between the ages of 30 and 60.The diseases associated with nucleoplasm fine granular and nucleoplasm coarse granular patterns are SLE, Sjogren's syndrome and MCTD. The diseases associated with nucleoli coarse granular and nucleoli fine granular patterns are Progressive systemic sclerosis, Progressive systemic sclerosis is a generalized disorder of connective tissue in which there is thickening of dermal collagen bundles, and fibrosis and vascular abnormalities in internal organs.

4. CONCLUSION

Disease specific and even disease associated auto-antibodies are biomarkers not only to confirm the diagnosis of the respective systemic auto Immune disease but also to diagnose the disease at very early stages or to diagnose the respective disease without the typical clinical manifestations. Another valuable use of auto-antibodies is that they facilitate an understanding of the complex pathogenesis of systemic autoimmune diseases. A confirmation of diagnosis in early stages is required if patients are to benefit from early therapeutic intervention.

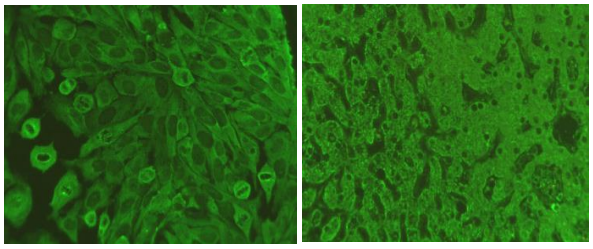

Figure 9
Cytoplasmic positive

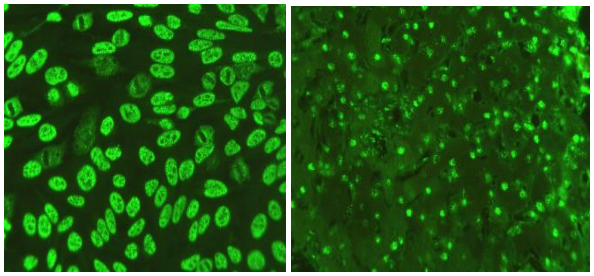

Figure 10
Nucleoli coarse granular

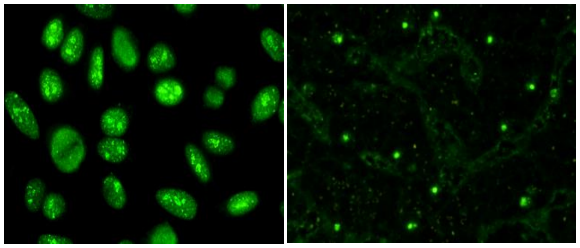

Figure 11
Nucleoli fine granular

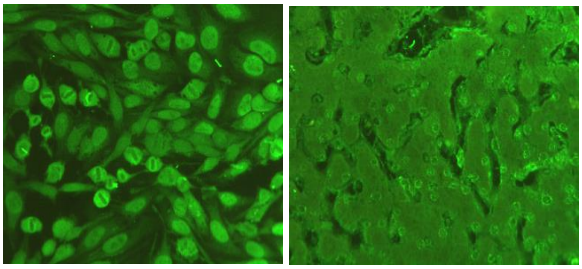

Figure 12
Nucleoplasm fine granular + Cytoplasmic positive

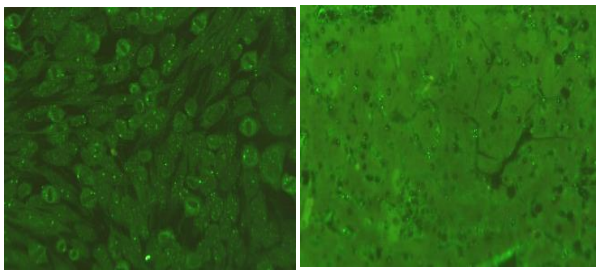

Figure 13
Cytoplasmic coarse granular + nuclear dots

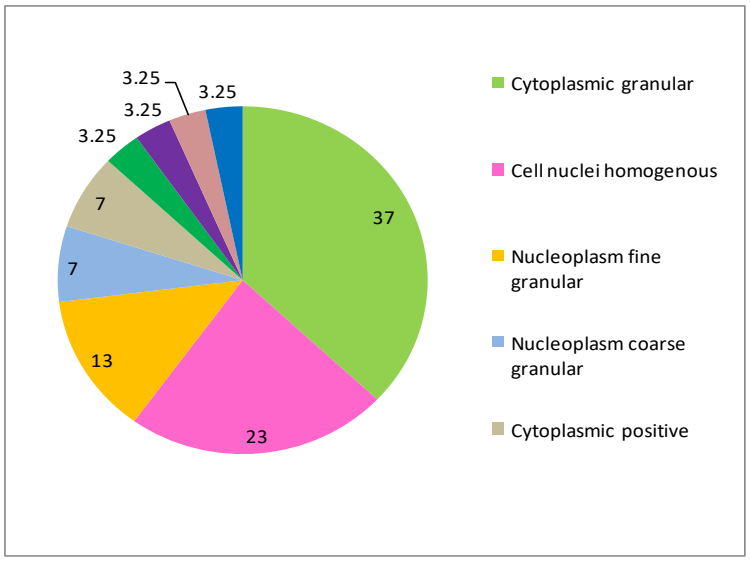

Figure 14
Prevalence of Auto antibodies

SUMMARY RESEARCH

1. Autoimmune diseases are conditions in which the immune system of the body treats its own tissues as foreign and tries to destroy them. The cause is unknown but many theories have been propounded to explain the development of these diseases. Heredity, stress, infections and chemical toxicity are some of the causes. Auto immune diseases affect 3-5% of the US population and their clinical presentations are very heterogeneous.
2. Presence of auto-antibodies are the common feature of an autoimmune disease produced by auto reactive Lymphocytes.
3. Our present study focuses on the use of indirect immuno fluorescence for the detection of auto antibodies in patients presenting with clinical symptoms. In the 50 samples examined in the present study, Polymyositis and Systemic lupus erythromatosus are more prevalent and are identified by Cytoplasmic pattern correlating with JO-1 and nuclear homogeneous pattern respectively. Thus emphasizing the usefulness of auto antibodies as reporter molecules in the identification of these autoimmune diseases.
4. Newer technologies like Fluorescent microsphere immunoassay, Peptide microarrays, Proteomics can be used as powerful approach to characterize the autoantigens which can lead to better understanding of the nature of auto immune diseases.

FUTURE ISSUES

To study the prevalence of different types of Auto immune diseases in Indian population**.**

DISCLOSURE STATEMENT

There is no conflict interest and financial support for the proposed research work.

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