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Full Length Research Paper

Evaluation of indirect immunofluorescence assay in patients with autoimmune diseases

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Antinuclear antibodies (ANA) are the hallmark of autoantibody production in autoimmune disease. ANA are directed against components of the cell nuclei such as DNA, histones, nucleoli and ribonucleoproteins. ANA detected by indirect immunofluorescence (IIF) is the method of choice and is regarded as the gold standard technique. 100 patients with autoimmune disease attending a tertiary care hospital at Chennai, India and ten healthy controls were included in the study. Detailed clinical, serological and biochemical analysis were done. Blood samples were analyzed for ANA by IIF using in-house mouse liver and in-house HEp-2 cell line substrate. The cells showing apple green fluorescence and a definable nuclear staining pattern were taken as positive and the intensity of fluorescence graded from + to ++++ in serial dilutions of the sera. IIF patterns like homogeneous (solid) rim (peripheral), speckled and nucleolar patterns were observed. ANA positivity by IIF for SLE is 92.7 and 97.4% on mouse liver and HEp-2 cells, respectively. Specificity of ANA by IIF is 80 and 90% for mouse liver and HEp-2 cell substrate, respectively. Although, many commercial assays are available for the detection of ANA, IIF by in-house methods still holds good and is justified in this research work.

Key words: Autoantibodies, indirect immunofluorescence, antinuclear antibodies, systemic lupus erythematosus (SLE).

INTRODUCTION

Autoimmune diseases are conditions in which the immune system damages specific organs or tissues that then result in systemic ill health. In most of the autoimmune diseases, it is thought that self-antigens provide the autoantigen drive for production of autoantibodies by B-cells, although the triggers may be exogenous factors. The cause is unknown but few of the risk factors proposed include genetic and epigenetic factors, complement deficiency and sex hormones. The various types of autoimmune diseases are systemic lupus erythematosus (SLE), Rheumatoid Arthritis, Systemic Sclerosis, Sjögren Syndrome, Juveline Idiopathic Arthritis, Hashimoto Thyroiditis, Graves disease, Polymyositis and Addison's disease, to name a few. The first description of ANA test began with the seminal discovery of the LE cell and LE cell phenomenon by Hargraves (1949). Although, these antibodies were considered a serological hallmark of SLE, they are also present in autoimmune disease sera. These may belong to any of the immunoglobulin isotype such as IgG. IgM, IgA, IgD and IgE and they can predate the onset of syndrome by several years (Eriksson et al., 2011). IIF is the method of choice for detection of ANA when compared to many novel techniques like commercial multiplexed ELISA (Showman et al., 2005) and in the diagnosis of autoimmune diseases like SLE (William, 2000).

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MATERIALS AND METHODS

1. Two techniques commonly used to detect autoantibodies are IIF and ELISA (Alem et al. 1987; Kavanaugh et al., 2000).

2. Direct immunofluorescence: In the direct method, biopsy of the affected tissues is stained with fluorescent conjugated antibodies to the autoantigens to be detected.

3. Indirect immunofluorescence: In the indirect method, serum samples are applied to normal tissues containing the relevant antigens. The bound antibody is detected by fluorescent conjugated anti-human immunoglobulin.

3. IIF is widely employed in rheumatology to detect and measure ANA in patients' sera (Kumar et al., 2009; Molden et al., 1984; Morris et al., 1973). When suitable substrate expressing the cellular antigen is allowed to react with sera containing specific antibodies, an antigen-antibody reaction takes place resulting in the localization of immune complexes at the site where the cellular antigen is located. Then the immune complex is traced with fluorescein labeled anti-human immunoglobulin. Different types of patterns of IIF staining correspond to reference sera that are known to contain specific autoantibodies (http://asc.dental.ufl.edu/ReferenceSera.html#text). Different substrates can be employed depending on the specificity of antibody which is to be detected. The relevant substrate antigens may be cryopreserved sections of rodent liver or human epithelioma cell line (HEp-2) cells (Sack et al., 2009). The different patterns like homogeneous rim, nucleolar, speckled were observed correlating with different subset of diseases (To and Petri, 2005).

Materials required

1. 100 samples of patients of various autoimmune diseases attending Rheumatology department, Government General Hospital.
2. Young healthy mouse obtained from animal house.
3. HEp-2 cell line maintained in the Virology Department.
4. Complement inactivated sera diluted from 1:20 to 1:160 in phosphate buffered saline (PBS).
5. HEp-2 cells were maintained in continuous culture in RPMI medium containing 10% fetal calf serum and 2% maintenance medium by subculture every 3-4 days.
6. Multi-spot slides were prepared.
7. Slides are sterilized by using dry heat at 160°C for 1 h.
8. 25 ml HEp-2 cell culture containing 5×10^4 cells/ml are dispensed into 90 mm diameter Petri dish.
9. Sterile multi-spot slides were immersed in the Petri dish and incubated at 37°C for 48 h in 5% CO_2. Periodically cell count is assessed with an inverted microscope.
10. When cells have grown to confluence, the slides were washed in PBS for 15 min and fixed in acetone:methanol (1:1) for 15 min.
11. Then slides are rinsed in distilled water for 15 min to wash off the fixative, air dried and stored in plastic bag at -20°C until required.

Methodology

Patient sera were placed in water bath at 56°C for 30 min for complement inactivation:

1. Complement inactivated samples were serially diluted 1/20, 1/40, 1/80, 1/160 in PBS. However, samples were tested at a screening dilution of 1:40 rather than 1:20 since HEp-2 cells are more sensitive.
2. 50-100 µl of diluted sera was added to individual wells of the HEp-2 cell substrate and incubated at 37°C for 20-25 min in a moist chamber.
3. After incubation, the slides were washed with 3 changes of PBS in troughs.
4. AHIG FITC conjugate (diluted 1/50) in PBS was added on the slides and incubated at 37°C for 20-25 min.
5. The slides were washed in PBS for 3 times.
6. The slides were covered with a drop of glycerol saline, cover slips applied and viewed under a fluorescence microscope.

Interpretation

1. Apple green fluorescence of nuclei is positive.
2. Cells with black or yellow nuclei are negative.
3. Grading of fluorescence was graded from + to ++++ based on the intensity of fluorescence.

While interpreting the slide, pattern grading and titres are taken into account. Titre of ++ and above in a titre of 1:80 is taken as positive for SLE according to our lab standards.

Patterns observed in indirect immunofluorescence assay

Homogeneous (diffuse): The nuclei are stained throughout without any loss of intensity at the periphery. The nucleoli are not visualized as a distinct organelle, and there is no differentiation of the nuclear membrane. All nuclei appear to be stained to the same intensity. Sera showing homogeneous nuclear staining have antibodies that react primarily with double stranded DNA and/or deoxynucleoprotein.

Rim (Peripheral): The nuclear membranes of intact nuclei are more intensely stained than central areas. Any stringy fragments of nucleoprotein extruding from traumatized nuclei are also intensely stained. Sera showing peripheral nuclear staining contain antibodies against antigenic targets like nuclear envelope components.

Speckled: The nuclei show minute "specks" of fluorescence scattered throughout the nucleoplasm except for the nucleolar aea; these points of fluorescence tend to diminish in number at the nuclear periphery. Sera showing speckled nuclear staining are commonly correlated with antibodies specific for certain saline or phosphate buffer extractable nuclear antigens (Sm and RNP).

Nucleolar: The nucleoli are uniformly stained and appear sharply demarcated from the unstained nucleoplasm. .The antigens that account for nucleolar fluorescence are defined to be Scl 70, Nor 90, RNA polymerase, fibrillarin, PM/Scl.

Mixed pattern: This consists of mixture of several patterns such as Rim, Nucleolar, Speckled and Homogeneous.

RESULTS AND DISCUSSION

Various autoimmune disease groups (N=100) and ten healthy controls were tested for ANA (Table 1). In clinical studies of patients, it was found that the presence of autoimmune disease and their symptoms are not found in

Table 1. Breakup of disease groups.

Disease groups	Total number of patients (n=100)	Percentage of patients
SLE	39	39
Rheumatoid Arthritis	25	25
Javeline Idiopathic Arthritis	10	10
Systemic Sclerosis	14	14
Sjögren Syndrome	5	5
Polymyositis	5	5
MCTD	1	1
Others (Gout)	1	1

Table 2. Sexwise distribution of disease groups.

Diseases	Female (n-56)	Male (n-44)
SLE	34	5
Rheumatoid Arthritis	10	15
Juvenile Idiopathic Arthritis	3	7
Systemic Sclerosis	3	11
Sjögren Syndrome	2	3
Polymyositis	3	2
MCTD	1	0
Gout	0	1

Table 3. ANA positivity on mouse liver substrate.

Disease groups	Total number of patients	Dilutions of sera positive number				Dilutions of sera positive percentage			
		1/20	1/40	1/80	1/160	11/20	1/40	1/80	1/160
Systemic Lupus Erythematosus	39	36	36	36	35	92.3	92.3	92.3	89.78
Rheumatoid Arthritis	25	5	4	4	3	20	16	16	12
Juvenile Idiopathic Arthritis	10	2	2	2	1	20	20	20	10
Systemic Sclerosis	14	3	3	2	2	21.42	21.42	14.28	14.28
Sjögren Syndrome	5	2	2	1	1	40	40	20	20
Mixed connective tissue diseases	1	1	1	1	1	100	100	100	100
Polymyositis	5	1	1	1	1	20	20	20	20
Gout	1	0	0	0	0	0	0	0	0
Healthy controls	10	2	2	0	0	20	20	0	0

young healthy individual of ages 1-20. With increasing in age, there is an increased incidence of autoantibodies in both “normal” males and females. It was observed that the increased incidence is found in females of reproductive age than in males (Table 2).

The results of ANA detection by in-house method on mouse liver substrate are depicted in Table 3. ANA positivity was found to be 92.3% in SLE and 100% in the case of mixed connective tissue disease (MCTD) (100%). Healthy controls were positive for ANA in low titres. The disease groups and ten health controls were tested for ANA on HEp-2 cell substrate and the results are depicted in Table 4. In this, 97.44% positivity was observed in SLE and 100% for MCTD.

Using both the diagnostic techniques, the typical fluorescence patterns that were observed on liver cells were 27% of homogenous, 23% of speckled (fine and coarse), 7% of rim, 2% of nucleolar and mixed patterns, 41% (Table 5). On HEp-2 cell substrate, all IIF patterns were appreciated better than mouse liver substrates

Table 4. ANA positivity of HEP_2 cell substrate.

Disease groups	Total number of patients	Dilutions of sera positive number				Dilutions of sera positive percentage			
		1/20	1/40	1/80	1/160	1/20	1/40	1/80	1/160
Systemic Lupus Erythematosus	39	38	38	38	37	97.44	97.44	97.44	92.3
Rheumatoid Arthritis	25	8	7	6	6	32	28	24	24
Juvenile Idiopathic Arthritis	10	4	4	3	3	40	40	30	30
Systemic Sclerosis	14	5	5	4	3	35.71	35.71	28.57	21.42
Sjögren Syndrome	5	3	2	2	2	60	40	40	40
Mixed connective tissue diseases	1	1	1	1	1	100	100	100	100
Polymyositis	5	2	2	2	2	40	40	40	40
Gout	1	0	0	0	0	0	0	0	0
Healthy controls	10	1	1	0	0	10	10	0	0

Table 5. Comparison of ANA patterns on mouse liver substrate and HEP_2 cell substrate.

Patterns	Mouse liver substrate percentage	HEp-2 cell substrate percentage
Homogeneous	27	37
Speckled	23	26
Rim	7	13
Nucleolar	2	5
Mixed	41	19

Table 6. Statistical analysis of ANA on mouse liver substrate in diagnostic groups.

Diseases	Sensitivity (in%)	Specificity (in%)
SLE	92.3	80
Rheumatoid Arthritis	80	80
Juvenile Idiopathic Arthritis	80	80
Systemic Sclerosis	78.7	80
Sjögren Syndrome	60	80
Mixed connective tissue disorders	100	80
Polymyositis	20	80

(Table 5).

ANA detection by mouse liver substrate has the advantage of simplicity and cost effectiveness. It still holds good for labs with limited infrastructure. Since the nuclei are smaller, certain patterns may be missed by these methods. On the other hand, HEp-2 cells are rapidly proliferating, displaying a much higher portion of mitotic figures and so certain patterns are better appreciated with HEp-2 substrates like anti-SSA, anti-SSB anti-centromeric patterns. Positive ANA can be observed in elderly healthy individuals in lower titres.

The results were analyzed using SPSS package and interpreted. Tables 6 and 7 illustrate the sensitivity and specificity patterns of these two techniques. Sensitivity of mouse liver substrate for autoimmune disease is 81.8% and specificity is 80%. Sensitivity of HEp-2 cell substrate for autoimmune disease is 88.8% and specificity is 90%. Sensitivity of mouse liver substrate for SLE is 92.3% and specificity is 80%. Sensitivity of HEP2 cell substrate for SLE is 97.4% and specificity is 90%.

Conclusion

IIF for ANA (FANA) provides rapid but highly sensitive method for ANA detection. The results are reported by four parameters like pattern, titre grading and the type of substrate used. Any pattern of positive fluorescence at a

Table 7. Statistical analysis of HEP_2 cell substrate in diagnostic groups.

Diseases	Sensitivity (in%)	Specificity (in%)
SLE	97.44	90
Rheumatoid Arthritis	68	90
Juvenile Idiopathic Arthritis	60	90
Systemic Sclerosis	64.28	90
Sjögren Syndrome	40	90
Mixed connective tissue disorders	100	90
Polymyositis	20	90

titre of 1:40 and more is taken as positive for autoimmune disease. For SLE, 1:80 is taken as the cut-off titre. The negative ANA excludes SLE, MCTD. Very rarely, ANA can be negative in SLE. It has been concluded that, mouse liver ANA in-house test is inexpensive though animal has to be scarified. HEp-2 cell maintenance is an expensive procedure and stringent precautions need to be taken for preparing in-house HEp-2 substrate and the patterns are better appreciated using this cell line for detection of ANA.

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