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Immunology

RECENT ADVANCES IN LABORATORY DIAGNOSIS OF SYSTEMIC AUTOIMMUNE DISEASES.

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ABSTRACT **Introduction:** Autoimmunity is a condition in which the body's own cells which are immunocompetent and the antibodies, acts against its own self-antigen which will result in structural and functional damage to the body. The diseases caused by this phenomenon are called Autoimmune diseases. Autoimmune diseases are more commonly seen in females. Hundred thousands of individuals in the Western countries are affected. The diagnosis is made from the clinical presentation of the patient with which a differential diagnosis is made, following which various tests both phenotyping methods and genotyping methods are carried out to conclude the final diagnosis.

Conclusion: The genotyping methods play the most important role in the laboratory diagnosis of systemic immune diseases.

KEYWORDS : Autoimmune diseases, Flow Cytometry TOF-MS, Genotyping methods, HLA typing, Multiplexed Immunoassays.

INTRODUCTION:

Normally immune system develops antibodies against foreign substances like antigens when they enter the body. At times, body's immune system behaves in a different way by producing antibodies against its self antigens which results in autoimmunity. Early in the 19th century, Paul Ehrlich coined the term *'horror autotoxicus'*, when he realized that sometimes the immune system doesn't behave in the intended way.

Autoimmune diseases are a condition in which a host immune system is activated against its self components contributing to the disease pathogenesis. There are around 80 immune diseases resulting from mild to disabling severity (1).

EPIDEMIOLOGY:

Autoimmune diseases are one among the leading causes of death in the young and middle aged woman in Western countries. The prevalence and the incidence rates vary among the autoimmune diseases. Ex., Systemic Sclerosis is seen < 1 newly diagnosed case to > 20 cases of adult onset in Rheumatoid arthritis (RA) per 100,000 person years. Prevalence: <= 5/100,000 to > 500 / 100,000 the former being chronic active hepatitis, uveitis to the latter of Grave's diseases, RA, thyroiditis (2).

Most of the autoimmune diseases [Systemic Lupus Erythematosus, Sjogren's syndrome, Systemic Sclerosis] are more common in female. Age predisposition differs with various autoimmune diseases. Ex., Type 1 Diabetes mellitus is more common in childhood & adolescence (2).

Genetic Factors:

i. Mutations in short arm of Chromosome 6 – Human leukocyte antigen [HLA] or major Histocompatibility Complex [MHC] is also another factor responsible for Autoimmune diseases.

ii. Other genes associated with development of autoimmune diseases are

· AIRE gene: It is an autoimmune regulator present in Chromosome 21 and mutation in this gene leads to failure in expressing self-antigens correctly in the thymus which prevents negative selection [clonal deletion] of thymocytes and autoreactive T cells escapes into the periphery leading to APECED syndrome.

· FOXP3 gene: Defects in this gene leads to T regulatory cells absence which are involved peripheral control of T cell reactivity in the autoimmune mechanisms leading to IPEX syndrome.

· CTLA 4 gene: Polymorphism in this gene is associated with autoimmune thyroiditis. i.e., Grave's disease and Hashimoto's thyroiditis.

· PTPN 22: This T cell signaling gene encodes a tyrosine phosphatase responsible for down reglating T-cell activation (3).

Other factors:

i. Superantigen causes unregulated T cell activation & by stander damage.

Ex., *Klebsiella pneumoniae* & Zika virus are linked with Ankylosing spondylitis & Guillian Barre Syndrome respectively also following Reactive arthritis, following Salmonella and Yersinia infection (4,5).

ii. Drugs: Procainamide, hydralazine is proved to induce autoimmunity in some individuals (6).

MECHANISMS OF AUTOIMMUNTIY :

There are three mechanisms which are involved in maintaining the unresponsiveness to self antigens (1):

I. Sequestration of self antigen.

ii. Generation and maintenance of central and peripheral tolerance.

iii. Regulatory mechanisms.

When there is breach in these mechanisms, it will result in autoimmunity.

Table 1: Mechanisms Of Autoimmunity

EXOGENOUS:	
A	Molecular mimicry
	Superantigenic stimulation
	Microbial adjuvanticity
ENDOGENOUS:	
A	Altered antigen presentation
	Loss of immunologic privilege
	Presentation of novel or cryptic epitopes
	Alteration of self antigen
	Enhanced function of antigen presenting cells
B	INCREASED T-CELL HELP
	Cytokine production
	Co-stimulatory molecules
C	INCREASED B-CELL FUNCTION
D	APOPTOTIC DEFECTS
E	CYTOKINE IMBALANCE
F	ALTERED IMMUNOREGULATION

EXOGENOUS:

i. MICROBIAL ADJUVANTICITY:

The bacterial endotoxin, RNA / DNA possessed by microbes have adjuvant like effects on the immune system which interferes with the normal tolerance mechanisms and result in autoimmune disease.

ii. SUPERNTIGENIC STIMULATION:
Super-antigen from the bacteria /virus / fungus will bind to the variable 'β' region on the T-cell receptor and induces production of non-specific cytokines which at times leads to autoimmunity. Ex., *Staphylococcus* Toxic Shock Syndrome.

iii. MOLEULAR MIMICRY:
Cross reactivity between a microbial product and a self-antigen due to similarity between the both, leads to activation of the auto-reactive lymphocytes.

Ex.: Acute Rheumatic Fever: 'M' protein of *Streptococcus pyogenes* is similar to myosin, laminin and other matrix proteins which results in antibody production against the matrix proteins. When such auto-antibodies in heart gets deposited it results in carditis and if deposited in brain results in Syndenham's chorea.

ENDOGENOUS:
I. Loss Of Immunological Privilege:
Privileged sites like brain and eye whose proteins are within the sites and are not exposed to the immune system during T-cell and B-cell development and maturation. When there is any damage like trauma / inflammation these proteins are exposed to the immune system and become the targets of immune assault by activated T-cells. Ex., Multiple Sclerosis, Sympathetic ophthalmia.

II. Defects In Apoptosis:
Defect in clearance of apoptotic material will result in autoimmunity and autoimmune diseases. Ex., SLE.

III. Polyclonal B-cell Activation:
Polyclonal B-cell activation is due to intense stimulation of T lymphocytes which produce non- specific signals and thereby result in multiple auto-antibodies production. Ex., Anti-nuclear antibodies, anti-erythrocyte antibodies are produced during chronic graft versus host reaction (1)

Classification:
Autoimmune diseases are classified as either organ specific or organ non-specific diseases depending on the site of pathogenesis.

Organ Specific:
I. Endocrine: Grave's disease, autoimmune thyroiditis.
II. Gastrointestinal: Pernicious anemia, Celiac disease.
III. Skin: Pemphigus, Dermatitis herpetiformis

ORGAN NON-SPECIFIC:
I. SYSTEMIC : SLE, Scleroderma, Sjogren's syndrome, Dermatomyositis.
II. OTHERS : Myasthenia gravis, Autoimmune hemolytic anemia
In this article we will be discussing about the Systemic Autoimmune diseases in detail.

SYSTEMIC AUTOIMMUNE DISEASES
1)SYSTEMIC LUPUS ERYTHEMATOSUS [SLE]:
It is a systemic autoimmune disease, characterized by inflammation in multisystem organs viz. joints, skin and vasculature which is mediated by tissue binding auto-antibodies and immune complexes (1,7).

EPIDEMIOLOGY:
SLE is more common in females rather than in males, the ratio being 1.9: 1. The prevalence of SLE is 6% worldwide (2).

GENETIC FACTORS:
The genes make the individual more susceptible for developing SLE in future. Few such genes are STAT-4, HLA-DR, PDCD-1 (1).

OTHER FACTORS:
Few other factors which also predispose an individual to develop SLE are ultra-violet light, gender, Epstein-Barr virus, silica dust, smoking etc.,

PATHOLOGY:
The susceptible genes and the environmental factors, interact to form abnormal immune response which is different among each patients with SLE.

Self-antigens like nucleosomal DNA/ protein, RNA/protein in Sm, Ro, La, phospholipids which are present on the surface blebs of the apoptotic cells to which auto-antibodies are formed, which together form immune complexes and result in inflammation and disease develops. The immune complexes get deposited in different sites and results in clinical presentation like arthritis, nephritis when the complexes deposits in synovial space and glomerular basement membrane respectively (8).

Auto-antibodies are developed against various antigens like DNA, histone, Sm, RNP, SS-A, SS-B, nucleolar antigens.

CLINICAL FEATURES:
The clinical symptoms include malar rash, arthritis, nephritis, carditis and leucopenia.

2) RHEUMATOID ARTHRITIS [RA]:
It is a chronic inflammatory disease of unknown etiology, most commonly presents with symmetric, peripheral polyarthritis which is always associated with extra-articular symptoms (1).

EPIDEMIOLOGY:
It is the most common autoimmune disease affecting females belonging to 4^{th} to 6^{th} decades of life. The female are more susceptible to RA more than male the ratio being 3.35:1 (2).

GENETIC FACTORS:
HLA-DR1 is more important gene when identified in an individual, it denotes that the individual is more susceptible to have RA in the future (8).

OTHER FACTORS:
Infections like Epstein-Barr virus, Mycoplasma, Parvo B19 virus will make the individual susceptible to develop RA (1).

PATHOGENESIS:
When a genetically susceptible individual is triggered with any environmental factors like various infections will result in immune response leading to autoimmunity. Macrophages are responsible for synovial inflammation in the rheumatoid arthritis with T-cells playing critical role in initiation of synovitis. The synovial infiltrate also contains activated B-cells which is the site for the production of Rheumatoid factors which are specific antibodies for the Fc region of IgG. The immune complexes formed by IgG and RA factor can fix complement and enhances the inflammation. RA Factor is the reason for the extra-articular presentation of the disease (8).

Auto-antibodies are produced against altered gamma globulin, Rheumatoid arthritis associated nuclear antigen (RANA).

CLINICAL FEATURES:
Articular Manifestations:
i. Early morning joint stiffness lasting for more than a hour and reduced with physical activity.
ii. Small joints of hands and feet are involved first.
iii. Monoarticular, oligoarticular or polyarticular involving one, <= 4 joints or > 5 joints respectively.
iv. Wrist joint, metacarpophalangeal (MCP) joint and proximal interphalangeal (PIP) joints are most commonly involved.
v. The hallmark for RA is Flexor tendon tenosynovitis.

EXTRA-ARTICULAR MANIFESTATIONS:
i. Constitutional : Fever, fatigue, weight loss etc.,
ii. Nodules: Firm, non-tender subcutaneous nodules are seen. Mostly they are adherent to the underlying structures like periosteum, tendons or bursae.
iii. Pulmonary: Pleural disease is common in RA. Pulmonary function test shows restrictive pattern.
iv. Cardiac: Pericardial involvement is very common in RA followed by cardiomyopathy.
v. Vasculitis: Rheumatoid vasculitis presents with petechiae, purpura, digitsl infarcts, gangrene etc.,
vi. Hematologic: Normocytic, normochromic anemia, thrombocytosis are noted in RA.
vii. Lymphoma: Diffuse large B-cell lymphoma is the most common lymphoma seen in RA patients (1).

3) SYSTEMIC SCLEROSIS [SSc]:
It is a multisystem disorder of unknown etiology associated with vascular abnormalities, connective tissue sclerosis and fibrosis and auto-antibodies.

Discoid SSc is cutaneous form of systemic sclerosis with thickened skin (scleroderma) with multiple organs involvement (lungs, gastrointestinal tract, heart and kidneys) (1).

EPIDEMIOLOGY:
It is a sporadic disease and affects all races around the world. SSc also shows female predominance but are very common in reproductive age group and the prevalence reduces after menopause (2).

GENETIC FACTORS:
Family history is very important as SSc follows Mendelian inheritance pattern. These individuals are in high risk to develop SLA, RA. Genes which encode for Angiotensin Converting Enzyme (ACE), B-cell marker (CD19), chemokines (monocyte chemoattractant protein-1), interferon signaling mediators like STAT-4 and IRF-4 are associated with SSc. (1)

OTHER FACTORS:
Individuals who are affected with SSc will have plenty of auto-antibodies against human Cytomegalovirus and Topoisomerase-I in the serum. Exposure to Parvovirus, and other factors like silica , polyvinyl chloride are also associated with systemic sclerosis (1).

PATHOGENESIS:
When there is a vascular injury there is initiation of endothelial cell and platelets activation. This will recruit leukocytes like CD4+ and CD8+ T cells and activated B cells to the site which will secrete cytokines like TGF-β, CTGF and other chemokines. These B cells may initiate the production of auto-antibodies too. The released chemokines will induce fibroblast activation leading to collagen, connective tissue accumulation resulting in tissue fibrosis.

Auto-antibodies are produced against antigens like topoisomerase-I, centromere proteins, RNA polymerase-III etc (8).

VARIANTS:
- Diffuse Systemic Sclerosis.
- CREST Syndrome.

Clinical Features:
i. SKIN: Symmetric and bilateral skin thickening is seen.
ii. RAYNAUD'S PHENOMENON: It is an episodic vasoconstriction seen in the fingers and the toes of the patients with SSc, when exposed to cold, or any stress.
iii. OTHER INVOLVEMENT: Esophageal involvement, pulmonary fibrosis, pulmonary artery hypertension, and scleroderma renal crisis are seen in patients with systemic sclerosis.

4) SJOGREN'S SYNDROME:
Sjogren's syndrome is a chronic autoimmune disease of exocrine glands like salivary & lacrimal gland which are infilterated with lymphocytes and they slowly progress to result in xerostomia and dry eyes (keratoconjunctivitis sicca) .

There are two types of Sjogren's syndrome : Primary and Secondary.
PRIMARY SJOGREN'S SYNDROME: Sjogren's syndrome alone.
SECONDARY SJOGREN'S SYNDROME: It is associated with other rheumatic disease, systemic sclerosis, polyarteritis nodosa etc,.

EPIDEMIOLOGY:
This condition is more commonly seen in females of all age groups. The prevalence of primary Sjogren's syndrome is 0.5 – 1%. Secondary Sjogren's syndrome is seen in 30% of the individuals who suffer from autoimmune rheumatic disease (2).

GENETIC FACTOR:
The individuals possessing HLA-DQA1*0501 allele are more susceptible to be affected by Sjogren's syndrome (8).

PATHOGENESIS:
The hallmark of Sjogren's syndrome is lymphocytes infliteration in the exocrine glands. CD4+ is more predominant among the lymphocytes infilteration. There is expression of Class-II HLA-DR antigen on the salivary & lacrimal epithelial cells which interact with CD4+ lymphocytes which stimulates polyclonal 'B'- cells to produce auto-antibodies.

CD4+ T-cells secrete IL-2,IL-6,IL-10 which will result in autoimmune manifestations, lymphocytic proliferation. Extra- glandular manifestations like vasculitis results in immune complexes formation followed by complement activation (8).

CLINICAL FEATURES:
Xerostomia, keratoconjunctivitis sicca, xerotrachea are the glandular manifestations of Sjogren's syndrome.
Arthritis, Raynaud's phenomenon, lymphadenopathy, vasculitis are few of the extra-glandular manifestations seen in Sjogren's syndrome (1).

5)ANTI-PHOSPHOLIPID ANTIBODY SYNDROME [APLA]:
It is an auto-antibody mediated acquired thrombophilia characterized by recurrent arterial/venous thrombosis and / or morbidity during pregnancy due to presence of auto-antibodies against Phospholipid binding plasma protein(β-2 glycoprotein I) and Prothrombin. Lupus Anticoagulant (LA) another group of antibodies prolong clotting times (7).

Anti-phospholipid syndrome is classified into two types: primary and secondary.

Primary APLA Syndrome: It is associated with only APLA syndrome.
Secondary APLA Syndrome: It is associated with other autoimmune disease.

EPIDEMIOLOGY:
It is seen in 1-5% of general population. One-third of the patients suffering from SLE have antibodies for anti-phospholipid (3).

PATHOGENESIS:
Infection, oxidative stress, major physical stress, surgery or trauma are the events which initiates the binding of antibodies to the phospholipid binding protein. It results in increased apoptosis of endothelial cells and exposure of phospohlipids subsequently. These phospholipids binds with β-2 GPI / Prothrombin and results in neo-antigen formation which triggers the induction of anti-phopholipids. Thus formed Anti-phospholipids binds to the disrupted endothelial cells leads to the initiation of intra vascular coagulation and thrombus formation. Complement activation is another mechanism of APS resulting in fetal diseases (1).

CLINICAL FEATURES:
VENOUS THROMBOSIS: Deep vein thrombosis, pulmonary embolism, superficial thrombophlebitis.
ARTERIAL THROMBOSIS: Stroke, Transient ischemic attack, digital gangrene, myocardial ischemia.

Neurologic Manifestations: Migraine, Epilepsy, Chorea, Cerebellar Ataxia.
Renal Manifestations: Renal Artery or Venous Thrombosis.
Obstetric Manifesations: Pre-Eclampsia, Eclampsia.
Fetal Manifestations: Fetal Loss, Premature Birth.
Other Manifestations: Arthralgia, Arthritis, Thrombocytopenia, autoimmune hemolytic anemia (1).

6) DERMATOMYOSITIS:
It is a disorder involving skin, muscle and blood vessels and characterized by erythematous and edematous changes in skin and also associated with inflammation of muscles (8).

EPIDEMIOLOGY:
Incidence is more common in first decade of life and is most commonly seen in girls with female to male ratio being 5:1. In adults it is seen predominantly in 4^{th} to 6^{th} decade of life (2).

ETIOLOGY FACTORS:
The disease is more associated with scleroderma (1), mixed connective tissue disorder and penicillamine (7) therapy.

VIRAL FACTORS:
Parvovirus B-19 plays a major role in disease manifested in adults whereas Coxsackie-B virus is more importantly linked with childhood infection (7).

PATHOGENESIS:
Humoral immunity mechanisms are involved in dermatomyositis and results in microangiopathy and muscle ischemia.

Activation of complement C5b6789 membrane lytic attack complex triggers the release of pro-inflammatory cytokines and chemokines. These mediators induce the expression of Vascular and Intercellular Cell Adhesion Molecule (VCAM & ICAM) respectively which are present on the endothelial cells.

These adhesion molecules induce migration of activated lymphocytes cells to endomysial spaces in the endothelial cells which results in necrosis of the endothelial cells and micro-infarcts occur (1).

CLINICAL FEATURES:
Skin rashes-heliotrope rash, muscle weakness, erythema of the knuckles with raised violaceous scaly eruption, dilated capillary loops in the finger nails are seen.

Few systemic manifestations are also seen in dermatomyositis patients like subcutaneous calcification, interstitial lung disease, dysphagia and Raynaud's phenomenon (2).

CLINICAL APPROACH:
Approach to a patient suspecting to have an autoimmune disease requires a detailed history taking, complete physical and systemic examination. A differential diagnosis can be made from the history and examination findings and the final diagnosis can be achieved from specific investigations reports.

CLINICAL HISTORY: The clinical history should contain the history of exposure to ultra-violet radiation, drug intake (Ex., Penicillamine), recent viral infection (Ex., Parvovirus B19) or history of chemicals (Ex., Smoking).

PHYSICAL EXAMINATION: Physical examination should include looking for malar rashes (SLE), heliptrope rash (Dermatomyositis). Rheumatoid nodules (RA) in elbow, discoloration of tips of fingers or toes (Raynaud's phenomenon).

LABORATORY INVESTIGATIONS:
The laboratory investigations can be divided as non-specific and specific investigations. Non-specific tests are those which helps in finding any abnormality seen in the patient's sample, but will not help in coming to a final diagnosis. Ex., Complete Blood Count, Coomb's test, RA factor.

There are radiologic investigations like parotid sialogram done for Sjogren's syndrome, X-ray for rheumatoid arthritis patients etc.

SPECIFIC INVESTIGATIONS:
The specific investigations are those which help in coming to a final diagnosis.

The hallmark of autoimmune diseases is the presence of auto-antibodies. These can be detected either as a biomarker to look for the presence of disease or can also be pathogenic in few patients. So detection of such auto-antibodies plays an important role in the diagnosis of autoimmune diseases.

The investigations done for achieving the final diagnosis of an autoimmune disease are discussed below.

ANTI-NUCLEAR ANTIBODIES DETECTION BY INDIRECT IMMUNOFLUORESCENCE TECHNIQUE:
The immunofluorescence technique for anti-nuclear antibodies detection, denotes specific subtype based on the nuclear or cytoplasm was designated in 1957.

HISTORY OF ANTI NUCLEAR ANTIBODIES:
'LE' cell preparation test was the first anti nuclear antibodies (ANA) detection method in 1947 by Hargraves. This test was used for the diagnosis of SLE (9).

PROCEDURE:
ANA detection by IIF is performed using HEp-2 cells. The cells were coated onto coverslips, fixed with acetone, cut into fragments and glued onto microscope slides. Serum samples are diluted to 1:100 dilution and incubated with HEp-2 cell substrate for 30 minutes at room temperature.

Table 2: List Of Autoantibodies To Be Detected For Specific Diseases.

DIESEASE	AUTO-ANTIBODIES DETECTED
ORGAN-SPECIFIC DISEASES:	
Thyroidits	Thyroglobulin (TGA), Thyroid peroxidase (TPO).
Type-I Diabetes mellitus	Insulin and glutamic acid decarboxilase auto-antibodies.
Primary biliary cirrhosis	Anti- mitochondrial auto-antibodies .
SYSTEMIC AUTOIMMUNE DISEASES:	
SLE	Anti-dsDNA, anti-Sm, anti-ribosomal P auto-antibodies .
Scleroderma	Anti-topoisomerase-I (Scl-70) auto-antibodies.
RA	Anti-citulline-modified proteins antibodies.
Sjogren's syndrome	Anti-SS-A/Ro, anti-SS-B/La auto-antibodies.
Mixed Connective Tissue Disorders	Anti-U1-RNP, anti-PM-Scl auto-antibodies.
Dermatomyositis	Anti-Jo-1 auto-antibodies.

After washing with buffer, the slides are incubated for 30 minutes with goat anti- human IgG conjugated with fluorescein isothiocyanate, propidium iodide for counter staining to label specifically bound antibodies.After second washing step and embedding, the slides were subjected to immunofluorescent microscopy.The sera with antibody titer $\geq$1:100 is positive.

Based on this, different types (viz) homogenous, speckled, nucleolar, nuclear dots, centromeres, cytoplasmic, peripheral patterns are reported (10).

II ENZYME IMMUNOASSAY:
Enzyme immunoassay is a term which describes the tests which uses enzyme-substrate system to detect either antigen or antibody or hapten from a specimen.

PROCEDURE:
PREPARATION OF ANTIGENS:
- Prepared from nuclear extracts of human epithelial type-2 cells (HEp-2).
- Prepared from purified nuclear antigens.
- Prepared from recombinant antigen.

Blood sample will be collected from the patient, serum is separated and the procedure will be performed according to the manufacturers' description. HEp-2 ANTI NUCLEAR ANTIBODIES ENZYME IMMUNO-ASSAY is an automated method with high reproducibility and internal calibration as a basis for standardization (11).

LIMITATIONS:
- The specificity of EIA depends on the quality of the antigens prepared for the procedure.
- The HEp-2 preparation of antigens has very minimal use in the techniques as they will not be pure and reproducibility will also be minimal.

III MULTIPLEX IMMUNOASSAY:
It is the most widely used method now. This assay detects multiple auto-antibodies in a single test at the same time (11).
Various techniques with different principles are used.
a. Microarray based assay.
b. Bead-based assay.
c. Proteomic's technology

a) MICROARRAY BASED ASSAY:
Line – Blot Assay:
Principle:
The line-blot immune-assay is a type of multiplexed immunoassay. This assay detects different types of auto-antibodies simultaneously at a same time.

Procedure:
Recombinant antigens are used in this assay. These different types of antigens are immobilized in a nylon test strip in a straight line. When a serum suspected to have the auto-antibodies in it, is added to the test strip and incubated. They form straight lines when they bind against their specific auto-antigen in the strip. The bounded antigen antibody complex is visualized using a color detection system depending on the

enzyme used in the strip.

RESULT:
The intensity of the colored lines formed are compared with the cut off lines, thereby reporting of results are done.

- **Sandwich Immunoassay:**

This method helps in detectio of multiple auto-antibodies simultaneously with different auto-antigens using sandwich immunoassay technique. Here, again the auto-antigens are immobilized on a microarray along with the reference proteins. The serum is added to the microarray and incubated. Then either a chemiluminescence or fluorescence tagged labeled secondary anti-human antibody will be added and the bound auto-antibodies are detected.

b)BEAD-BASED ASSAY:
This method recruits flow-cytometry for the analysis of anti-nuclear antibodies. The principle used here is microsphere based fluorescent assay.

PROCEDURE:
Synthetic microspheres made up of polystyrene are labeled with two different fluorochromes in a different proportion. Each fluorochrome will have any 10 possible levels of intensity of fluorescence, with a total of 100 different types of intensities of spectrum. The antigens and the auto-antibodies are bound together in microspheres. Each of the 100 microspheres will be differentiated from each other by the specific intensity of fluorescence they possess. Each microsphere will carry a specific immobilized antigen to a single auto-antibody based on the intensity of the fluorescence they carry. A green laser is used to excite the external reporter fluorescence to quantify the specific reaction related to the specific auto-antibody.

Commercially available flow cytometry can detect as many as nine anti-nuclear antibodies simultaneously.

The accuracy, reliability and the specificity is similar to that of enzyme immunoassays.

LIMITATIONS:
Quantitative calibration of the different auto-antibodies cannot be calculated.

c) PROTEOMIC'S TECHNOLOGIES:
From a given clinical samples like body fluids or cells or tissues, proteomics are able to detect new disease biomarkers. Analytical and clinical validation along with implementation of novel diagnostic or therapy related markers are the main aims of proteomics' technologies.

PROCEDURE:
This technology includes antigen microarray platforms which studies the immune response against foreign and the self antigens which may be involved in the development of the autoimmune disease or its progression. This technique allows the detection of auto-antibodies directed against any number of antigens like nucleic acids, peptides, proteins etc. Depending on the antigen to be detected the molecular probe is taken. Molecular probes can be either monoclonal or polyclonal. The bound molecules can be detected by using a fluorescent labeled secondary antibody. Then the incubated chips are read by the scanners depending on the technology used.

IV HUMAN LEUKOCYTE ANTIGEN (HLA) TYPING:
Human Leukocyte Antigens complex are the Major Histocompatibility Complex coding proteins which are present on the surface of all the nucleated cells in human body.

The Human Leukocyte Antigen complex coding for Major Histocompatibility Complex proteins are present in the short arm of Chromosome 6. The genes coding for the Major Histocompatibility Complex genes are clustered in three different regions called as MHC Region I, II and III which encodes for their proteins specific for their regions. Many HLA alleles are associated with increase in susceptibility to some diseases.

Table 3 : HLA Alleles And The Associated Diseases.

HLA Allele	Disease associated
HLA-B27	Ankylosing arthritis.
DR-2	Multiple sclerosis
DR-3	SLE, Myasthenia gravis.
DR-4	Rheumatoid Arthritis

HLA typing is a specific test which will help in knowing the genetic predisposition of an individual towards autoimmune diseases. HLA typing can be done by phenotyping methods or the molecular typing methods.

PHENOTYPING METHODS:
- Micro-cytotoxicity.
- Mixed Lymphocytic Reaction

MOLECULAR TYPING METHODS:
- Polymerase Chain reaction (PCR) detecting HLA genes.
- PCR-RFLP [Restricted Fragment Length Polymorphism]
- PCR-SSOP [Sequence Specific Oligonucleotide Probing]
- PCR-SSP [Sequence Specific Primers]

The phenotyping methods are obsolete now.

PCR based analysis have major influence on knowledge about the molecular basis for autoimmunity. Identification of individual allelic sequences had helped in knowing susceptibility/ resistance to few of the most important autoimmune disease. It also helps in understanding the mechanism by which the autoimmune disease is triggered.

MOLECULAR TYPING METHODS:
The molecular typing methods are rapid and are the method of choice in most of the laboratories. It requires good quality of genomic DNA for the processing.

DNA extraction steps include
1. Collection of whole blood,
2. Using physical / chemical methods to lyse the cells,
3. Using detergents for removal of the membrane lipids,
4. Followed by protease treatment for denaturation of proteins
5. Removal of other cellular containments by washing,
6. Treating the sample with RNAse to remove the RNA,
7. Wash with buffer
8. Purified DNA is extracted.

I. PCR-RFLP [RESTRICTED FRAGMENT LENGTH POLYMORPHISM]
This technique detects the fragments of DNA from the gene or flanking sequences, which contains restriction sites specific to each alleles. The variations in any sequence between any two alleles can be identified with the help of restriction enzyme cleavage that gives products of different sizes.

The fragments which are amplified will undergo digestion process and the final products are examined using agar gel electrophoresis. Multiple pairs of PCR primers are used in this method.

USES:
Used in typing of alleles or a group of alleles at the DPB1, DRB1, and DQB1 loci (12).

II. PCR-SSOP [Sequence Specific Oligonucleotide Probing]:
It is a simple and rapid method in detecting sequence variation among the alleles at HLA class II genes. It utilizes short, non radioactively labeled probes to type the amplified DNA by PCR. This technique use primers that are complementary to regions of the second exon which are conserved among all allele, which helps in detecting most of the class-II loci.

PROCEDURE:
o It is done by dot blot method or reverse dot blot method.
o As the first step of PCR, amplification of the desired DNA is done using single pir of biotinylated primer which flank the whole of exon thereby all the alleles are amplified from the exon.
o PCR product is denatured and then added to a well containing nylon membrane with bound probe. The well is then incubated with hybridization buffer. Wash away the excess products. Again the wells are incubated with a conjugate and enzyme which helps in visualization of the products. Mostly the conjugate- enzyme used is streptavidin and horse radish peroxidase complex. This complex binds to the biotin which is labeled in the probes. When a substrate is added

then the color will change to blue.
o The test is interpreted by entering the band pattern into a computer program.

III. PCR-SSP [Sequence Specific Priming]:
This method includes matched oligonucleotide primers, which is devoid of 3' –end mismatches, and such primers are more efficient in the PCR reaction by using thermo-stable DNA polymerase. The primer pairs are designed as such that they can be hybridized to either single alleles or families of similar alleles.

The amplification of the primers takes place and they are sorted based on their sizes using agar gel electrophoresis, which can be visualized by staining with ethidium bromide or SYBR green and exposure to ultraviolet light, which is documented and the results are interpreted.

The result depends on the presence or absence of the specific PCR products in reaction tubes with the primers of known allele specificity. An internal positive control primer pair is included in each PCR (13).

ADVANTAGES OF MOLECULAR TYPING:
- It will not require viable cells.
- The samples need not be transported immediately to the laboratories.
- They are good for batch testing (PCR-SSOP).
- It can be semi-automated.

DISADVANTAGES OF MOLECULAR TESTING:
- It requires good quality of DNA.
- The sequence of allele must be known.
- Requires a degree of redundancy within the primers used.

RECENT ADVANCES:
As autoimmune diseases are becoming more prevalent worldwide, there are many studies which are still in progress, in identifying the individuals who are susceptible for developing autoimmune disease in the future; even before the development of clinical manifestations.

Among the recent advances being developed now, the study of immune phenotyping is also done. This technique includes comparison of the cells present in the immune system both at rest and following ligand-induced activation. The signals which initiate this process is measured either using phosphoepitope identification or intra-cellular cytokine production or by identifying the biomarkers of the disease.
The recent advances included are
- Phophoepitope flow cytometry.
- Cytometry by time-of-flight mass spectrometry.
- Multiplexed bead immunoassays for biomarkers of immunology.
- Stimulated cytokine assays.
- Intracellular cytokine assays.

1. PHOSPHOEPITOPE FLOW CYTOMETRY:
The signaling system in immune cells mostly recruit a common post-translational modification, phosphorylation, to propagate the activation signals. A single cell level approach to analyze and interpret the transduction of signals is given by phospho-specific flow cytometry (14).

STEPS:
i. Isolation,preparation and stimulation of cells of interest.
ii. Fixation and permeabilization of the cells are done.
iii. Staining for surface antigens and phosphor-proteins seen over the cells
iv. Multiparameter flow cytometry analysis is done (14).

USES:
- Based on the phosphor-signalling networks this technique is used to analyze the correlation between the phospho -signaling and disease outcome of few cancers like Acute Myeloid Leukemia (15).
- It detects the cancer cell heterogeneity within the patient cells (15).
- The technique is used to study the directional and statistical maps of phospho-signaling networks within a cell (16).

2. CYTOMETRY BY TOF-MS:
Flow cytometry are used to study the cell types based on the surface markers, which are either activated or inhibited by antigens or cytokines in the body or based on the cell fate and function.

Unlike conventional flow cytometry, the flow cytometry TOF-MS has 7 different lasers and is capable of analyzing 22 parameters at a same time. Instead of using flurophores, the flow cytometry TOF-MS uses isotopic ionic salts which are from rare earth isotopes. There are 50 isotopes approximately used in this CyTOF method.

It is expected that in future the CyTOF can measure nearly 100 parameters of a cell type (17).

USES:
- To know the immune cell type, the various cytokines produced by the cell, the various ion levels and the intracellular proteins can be estimated by flow cytometry.
- It is used in the diagnosis of the autoimmune diseases.
- It can also be used to study the response of the patient to the therapy and thereby the prognosis of the patient can also be determined.

3.RECENT ADVANCES IN MULTIPLEXED BEAD IMMUNOASSAYS:
The recent advances in this method is to study the resting levels of any cytokines and also the stimulated levels of cytokines.

PROCEDURE:
The sample can be either whole blood or peripheral blood mononuclear cell (PBMC) which is incubated overnight, with a mixture of stimuli which can be any cytokines or any mitogens. The supernatant is taken and tested using multiplex bead assay to look for any cytokines which are released due to the action of the stimuli over the cells that converts them from resting stage to activated stage of releasing some levels of cytokines. This will help in understanding the mechanism if there is any dysregulation in the physiological cell signaling pathways (18).

ADVANTAGES:
- It is simple and the tool of choice for studying human immunology.
- It is a confirmatory method of auto-antibodies like Anti-nuclear antibodies, anti-neutrophil cytoplasmic antibodies etc.
- A very high number of analytes are tested simultaneously at a same time.
- Immunoglobulins, complementary and acute phase proteins can also be tested using this technique.
- Even when high number of analytes are examined the sensitivity of the technique is the same.

4. INTRACELLULAR CYTOKINE ASSAYS:
Normally when cells are induced they release specific cytokines. The cytokine which is produced can be studied by intracellular staining technique. This study can be done even from a very small population of cells, which can induce a detectable response when they are stimulated by any specific antigen.

USES:
It is helpful in studying the auto-antigen specific T-cells whose frequency is very low in the blood compared to the cells which are infected with various bacteria and viruses (18,19).

5. ALKIDES TECHNOLOGY:
ALKIDES technology is a fully automated interpretation technique which is combined with VideoScan technology. It is the first technology which is used as both indirect immune-fluorescent screening and confirmatory methods in detecting an auto-immune serology (20).

PRINCIPLE:
Based on the inverse fluorescence microscopy with various fluorochromes, the intensity of the fluorescence is measured by the system. After which it interprets based on the basic staining patterns seen on the HEp-2 cells (like conventional Indirect Immunofluorescence method).

ALKIDES PLATFORM:
This contains of
i) Technical System and Composition: The system consists of an inverse fluorescence microscope with different objectives and filters which are changed automatically; a movable scanning stage, light emitting diodes, gray level camera and all connected to a computer with software to analyze the images and interpret and perform data analysis.

ii) Software Concept: There is a sequential, multi stage process which includes acquisition of image, segmentation; description and classification of the objects in the image, quality control in order to evaluate automatic Indirect Immunofluorescence method. DAPI channel helps in performing the adjustment of position of the slide and the dynamic auto-focusing of the specimen. The software which is already fed with the details of the patterns to be recognized, to calculate the refractive indices of the objects in the specimen.

USES:

Along with studying the anti nuclear antibodies, this technology helps in studying the anti-neutrophil cytoplasmic antibodies [ANCA] and anti-double stranded DNA auto-antibodies assessment from the fixed human neutrophils and *Crithidia luciliae* respectively.

ALKIDES technology is also capable of assessing γH2AX foci using cell-based Indirect Immunofluorescence test. [γH2AX foci is used for individual biological response for radiation dose evaluation of DNA double-strand breaks] (20).

CONCLUSION:

Among the Western population, the prevalence of autoimmune diseases is high. The most common among them are Rheumatoid arthritis (3). Most of the diseases have female predisposition the more common among them being Scleroderma (2). All these diseases can be ruled out by detecting the Anti-nuclear antibodies present in the serum of the individuals. The susceptible individuals can be ruled out by performing HLA typing methods. Recent advancements in the investigations will help in ruling out any number of autoimmunity related conditions in an individual using multiplex assays (11) even prior to the appearance of clinical signs. After diagnosing, treatment should be initiated. This includes immune modulators like suppression of the immune system viz. Corticosteroids or Monoclonal antibodies viz. Rituximab (anti-CD20), Infliximab (anti TNF- α).

Conflict Of Interest: NIL

REFERENCES:

1. Jameson, Fauci, Kasper, Hauser, Longo, Loscalzo. Harrison's Principles of Internal medicine. 20th ed. USA: The McGraw-Hill Companies;2018.Vol(2);Part 11;Chap:342-4,348-51,353-4,358:2451-88,2510-40,2546-62,2590-3.
2. GS Kooper, BC Stroehla.The epidemiology of autoimmune diseases,Vol:2;Issue:3,May 2003:119-25.
3. T Ian, G Spickett. Lecture Notes Immunology 6th ed A John Wiley & Sons,Ltd.,Publication 2010;Chap.12:146-7.
4. L Zhang et all.The association of HLA-B27 & *Klebsiella pneumoniae* in ankylosing spondylitis: A systemic review. Microb Pathog;2018 Apr;117:49-54.
5. I Ketz, B Gilberd, O Shovman. Zika autoimmunity and guillain-Barre Syndrome. Curr Opin Rheumatol;2019 June 24.
6. E Cornaccchia, J Golbus, J Maybaum, J Strahler, S Hanash, B Richardson. Hydralazine and Procainamide inhibit T cell DNA methylation and induce autoreactivity.J Immunol;1988 Apr 1;140(7):2197-200.
7. B Tony, B Stephen, C Neil, G Christopher. Rook's Textbook of Dermatology.7th ed. UK: Blackwell Publishing Ltd;2004.Vol.3;Chap:56; The Connective Tissue Diseases;56.1-56. 147.
8. D Ivan, L James. Anderson's Pathology. 10th ed. New Delhi: Elsevier;2014;Vol(1)26: Autoimmunity and Autoimmune diseases;591-614.
9. Hargraves M, Richmond H, Morton R. Presentation of two bone marrow components, the tact cell and the LE cell. Mayo clin Proc 1948;27:25-8.
10. M Fenger, A Wiik, Hoter-Madsen M, JJ Lykkegaard, T RozenFeld, MS Hansen,et all. Detection of Antinuclear Antibodies by Solid-Phase Immunoassays and Immunofluorescence Analysis. Clin Chem 2004;50:2141-7.
11. S Ilza. Laboratory diagnosis of autoimmune diseases-new technologies, old dilemmas. Biochemia Medica 2010;20(1):45-56.
12. G Ulf , A Marie . PCR-based HLA Class II typing Genome Res.1991;Vol(1):91-98.
13. B Mike, IW Kenneth. Rapid DNA typing for HLA-C using sequence – specific primers (PCR-SSP):Identification of serological and non-serologically defined HLA-C alleles including several new alleles-Tissue antigens 1994;43(1),7-17.
14. Schulz KR, Danna EA, Krutzik Po, Nolan GP. Single-cell phosphor-protein analysis by flow cytometry. Curr Protoc Immunol.2007; 8:8.17.1-8.17.20.
15. Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phodpho-protein networks in cancer cells. Cell. 2004; 118:217-228. [PubMed: 15260991]
16. Sachs K, Perez O, Pe'er D, et all. Causal protein-signaling networks derived from multiparameter single-cell data. Science. 2005; 308:523-529. [Pubmed: 18157122]
17. Holden T. maecker, garry P. Nolan and Charles G. Fathman. "New technologies for autoimmune diseases monitoring" Curr Opin Endocrinal Diabetes Obes. 2010 August ; 17(4): 322-28.
18. Suni MA, Picker LJ, Maino VC. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. J Immunol Methods. 1998; 212:89-98. [PubMed; 9671156]
19. Waldrop SL, Pitcher CJ, Peterson DM, et al. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J Clin Invest. 1997; 99:1739-1750. [PubMed: 9120019]
20. Annika Willitzki, Rico Hiemann, Vanessa Peters, Ulrich Sack, Peter Schierack, Stefan Rodger et al. Review article-New Platform Technology for Comprehensive Serological Diagnostics of Autoimmune diseases. Clinical & Developmental Immunology Vol.2012;1-8 [article ID 284740]