Laboratory investigation of rheumatic and connective tissue diseases

Introduction

The overlap of clinical features in many of the rheumatic and connective tissue diseases has always complicated the task of segregating disease entities. Some recent developments in immunopathology have helped refine the process, though clinical assessment remains the cornerstone of diagnosis.

General Principles

Abnormal immunological activity forms the basis of the autoimmune disease process, the end result of which is target organ damage – for example, the synovial membrane in rheumatoid disease. Most laboratory investigations are based on the detection of antibody, largely surrogate markers of tissue damage, analogous to high blood pressure and vascular disease. As well as antibody detection, basic investigations are also useful in the diagnosis and monitoring of disease activity – for example, CRP levels in rheumatoid arthritis (RA), though they are less useful in systemic lupus erythematosus (SLE).

INVESTIGATIONS

Rheumatoid Factor (RF)

Rheumatoid factors are auto-antibodies (usually IgM, though they can be IgG and IgA) directed against antigenic determinants on the Fc portion of IgG. Agglutination tests using IgG-coated particles (latex or erythrocytes) form the basis of most diagnostic tests. Sullivan Nicolaides Pathology uses a quantitative latex immunoturbidimetric assay for the detection of RF.

Interpretation

RF assays (particularly latex assays) are not specific for RA. RF may be found in a variety of acute and chronic inflammatory diseases (Table 1), most of which are associated with a broadly elevated level of gamma globulin. RFs are also found in low titre (5%) in normal individuals, with titre and incidence increasing with age. Positive RF is, however, a highly sensitive diagnostic tool for RA, with 80% of RA patients having positive results, though only 50% will be positive at time of diagnosis. Thus the frequency of positive results increases with the duration of the disease.

Table 1

<table>
<thead>
<tr>
<th>Diseases commonly associated with RF</th>
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<tbody>
<tr>
<td>Connective Tissue Disease</td>
</tr>
<tr>
<td>• Rheumatoid arthritis</td>
</tr>
<tr>
<td>• Systemic lupus erythematosus (SLE)</td>
</tr>
<tr>
<td>• Sjögren’s syndrome</td>
</tr>
<tr>
<td>• Scleroderma</td>
</tr>
<tr>
<td>• Mixed connective tissue disease</td>
</tr>
<tr>
<td>Other Immunological Diseases (associated with hyperglobulinaemia)</td>
</tr>
<tr>
<td>• Chronic active hepatitis</td>
</tr>
<tr>
<td>• Fibrosing alveolitis</td>
</tr>
<tr>
<td>• Myelomatosis</td>
</tr>
<tr>
<td>• Cryoglobulinaemia</td>
</tr>
<tr>
<td>• Sarcoidosis</td>
</tr>
</tbody>
</table>

Chronic Infections

• Infective endocarditis
• Tuberculosis
• Syphilis
• Brucellosis
• Parasitic infections

Acute viral infections and post vaccination

Neoplastic disease (especially after irradiation or chemotherapy).

At the present time, tests for CCP antibodies are performed via an ELISA-based test and reported in numeric terms. The magnitude of the antibody level has not been reliably associated with clinical data.

Antinuclear antibodies, antibodies to extractable nuclear antigens, and anti-double-stranded DNA antibodies

Auto-antibodies to a variety of cellular components are a frequent finding in connective tissue diseases. Antinuclear antibodies (ANA) were first detected when the LE cell phenomenon was discovered. This test was superseded by the ANA indirect immunofluorescence test (IIF). Human epithelial cell line (Hep2000) cells are used for screening for ANA. This is a modification of the previous Hep2-based ANA test so that SSA is also detected. Thus, with this test, a negative result excludes active SLE. Many different patterns of cellular staining can be seen using this technique and may indicate the specificity of the antibody (Table 3). Tests for antibodies to extractable nuclear antigen (anti-ENA) attempt to further identify these antibodies. Seven anti-ENAs are routinely tested for by ELISA — anti-RNP, anti-SSA(Ro), anti-Ro52, anti-SSB(La), anti-Sm, anti-Scl-70, and anti-Jo-1. A different method (Line immunoenzymoassay) is used to confirm results and to detect anti-PM-Scl antibodies if specifically requested. Anti-double-stranded DNA antibodies (anti-ds DNA) are assayed by FEIA and radioimmunoassay (Farr assay) if requested.

Interpretation

Standard testing for ANA provides a rapid screening for SLE and related connective tissue disorders. A low titre (40-160) ANA may have little or no clinical relevance, occurring in a wide range of diseases (Table 2), as well as in a low percentage of normal individuals, particularly the elderly and the pregnant. The ANA is positive, however, in 100% of patients with active SLE, in up to 90% of patients with RA, and in 50% of patients with scleroderma.

Cyclic citrullinated peptide (CCP) antibodies

Over the past five years, the importance of antibodies directed against citrullinated peptides in the development of RA has allowed the subsequent production of commercial assays. Citrulline is a non-standard amino acid formed during cellular aging. CCP antibodies are present in a significant number (up to 70%) of RA patients. The antibodies may be present in those with a negative rheumatoid factor, and are useful in combination with the RF assay. The antibodies are termed cyclic citrullinated as this is the form of the antigen used in the test; as the name suggests, these antibodies are directed against multiple citrullinated peptides. CCP antibodies are highly specific, though they are seen in psoriatic arthritis and in some acute viral seroconversions (EBV, CMV etc.).
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**Table 2**

Causes of positive immunofluorescence for ANA

- Connective tissue diseases
- Chronic liver diseases
- Organ-specific autoimmune diseases: Pernicious anaemia, Hashimoto’s thyroiditis, Myasthenia gravis, Fibrosing alveolitis
- Chronic tuberculosis and leprosy
- Lymphoma and other malignancies
- Old age (> 60 years) (low titre)
- Pregnancy (low titre)

Six distinct patterns of nuclear staining may be seen using the ANA technique and may indicate the origin of the antigen involved. They are homogeneous, speckled, nucleolar, rim and centromere and dense fine speckled.

- Homogeneous and rim patterns are usually associated with anti-ds DNA and antihistone antibodies.
- Speckled patterns may be associated with anti-RNP, anti-Sm, anti-SSA and anti-Scl-70.
- Nucleolar antibodies may be associated with anti-Scl-70.
- Dense fine speckled is associated with DFS-70.

**COMMENTS**

**Anti-ds DNA**

Anti-ds DNA is moderately specific for SLE — 71% of patients with anti-ds DNA have been found to have SLE. The incidence rises to 82% five years after first detection of anti-ds DNA. Anti-ds DNA levels often correlate with disease activity. A rise in titre may predict disease flare.

**Anti-SSA**

Anti-SSA is found with varying frequencies in most connective tissue diseases, but particularly in SLE and Sjögren’s syndrome.

**Sjögren’s syndrome**

- Anti-SSA is found in 25% of patients with secondary sicca syndrome, and in 80% with the primary disease, often in association with hypergammaglobulinaemia and high-titre IgM RF.

**ANA-negative SLE**

Occasionally patients with clinical features typical of SLE have a negative ANA by HEp2. Now HEp2000 cells are used, all active SLE patients will have a positive ANA screen. A hallmark of the previously called ANA negative SLE condition appears to be a photosensitive rash. Anti-SSA is found in this subset of SLE patients.

**Neonatal SLE**

- Placental transfer of anti-SSA from mother to foetus may result in a transient lupus-like syndrome. This may cause congenital heart block and thus all pregnant women with SSA antibodies should be referred for specialist pregnancy followup.

**Anti-RNP**

This antibody is present in many of the connective tissue diseases. The absence of anti-RNP virtually excludes the diagnosis of ‘classic’ MCTD. Anti-RNP is also found in SLE, and rarely in polymyositis/dermatomyositis and scleroderma.

**Anticentromere Antibodies and Anti-Scl-70**

Anticentromere antibodies are usually associated with the CREST syndrome and occur in low frequency in SLE, MCTD, and scleroderma. Anti-Scl-70 is found in scleroderma and at low frequency in the CREST syndrome. Seen rarely in SLE where it is associated with pulmonary hypertension.

**Anti-Sm**

Anti-Sm is a highly specific marker for SLE, though it occurs in only 5% of SLE patients. There is no agreement on associations between anti-Sm and various disease features (e.g. CNS involvement and Raynaud’s phenomenon).

**Anti-SSB**

These antibodies occur with high frequency in primary Sjögren’s syndrome, but can also be found in SLE and RA. Their presence together with anti-SSA antibodies increases the risk of neonatal lupus syndromes.

**Anti-Jo-1**

- Anti-Jo-1 are specific for a subset of patients with myositis and mild overlap features (Raynaud’s phenomenon, sicca syndrome), and who have the complications of interstitial lung disease. It has also been described in patients with malignancy-associated myositis. Anti-Jo-1 rarely occurs in patients with lung or skin disease without myositis.

**Antithistone Antibodies**

Antithistone antibodies are detected by line immuno-assay and are useful in evaluating patients who have SLE-like syndromes induced by various drugs (Table 4). Greater than 90% of such patients have antihistone antibodies. As antihistone antibodies are present with varying frequencies in other autoimmune diseases, the test is only useful in excluding drug-induced SLE.

**Anti-Proliferating Cell Nuclear Antigen (PCNA) Antibodies**

These antibodies are directed against the cyclin complex, which is involved in the regulation of cell division. They are found in SLE and in patients with chronic hepatitis B or C. In SLE, they have been associated with glomerulonephritis.

**Anti-PM/Scl Antibodies**

These antibodies are directed against a group of proteins collectively called the exosome, which is critical in RNA production. They are present in a subset of patients who have both scleroderma and inflammatory myositis (polymyositis), and in around 5% of patients with either disease.

**DFS-70 Antibodies**

Dense fine speckled antibodies are identified based on their immunofluorescence and can be confirmed by ENA. The presence of DFS-70 alone represents a low risk of an underlying connective tissue disease.

**Anti-Ribosomal Antibodies**

Anti-ribosomal antibodies are present in SLE, though they are not common (5% or less). Some have associated their presence with neuropsychiatric complications. They are screened for by ANA with a cytoplasmic pattern combined with nucleolar pattern suggesting their presence. Confirmation is by line immuno-assay.

**Table 4**

Drugs implicated in drug-induced SLE

<table>
<thead>
<tr>
<th>Degree of Risk</th>
<th>Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Antihypertensives</td>
</tr>
<tr>
<td></td>
<td>Hydralazine</td>
</tr>
<tr>
<td></td>
<td>Antiarhythmics</td>
</tr>
<tr>
<td></td>
<td>Procanamide</td>
</tr>
<tr>
<td>Moderate</td>
<td>Anticonvulsants</td>
</tr>
<tr>
<td></td>
<td>Hydantoin; phenytoin</td>
</tr>
<tr>
<td></td>
<td>Succinimides: ethosuximide</td>
</tr>
<tr>
<td>Low</td>
<td>Antithyroid drugs</td>
</tr>
<tr>
<td></td>
<td>Thiourea derivatives</td>
</tr>
<tr>
<td></td>
<td>Psychotropic drugs</td>
</tr>
<tr>
<td></td>
<td>Phenothiazine derivatives</td>
</tr>
<tr>
<td>Undefined</td>
<td>Oral contraceptive agents</td>
</tr>
<tr>
<td></td>
<td>Oestrogen-progestin combination</td>
</tr>
<tr>
<td></td>
<td>Sulphonamides</td>
</tr>
</tbody>
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Table 3 Specificity of defined ANA in various disorders

<table>
<thead>
<tr>
<th>ANA Specificity</th>
<th>ANA* Pattern</th>
<th>Percentage of patients having antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds DNA**</td>
<td>HIR</td>
<td>SLE: 71, RA: 13, 1*SS: 18, MCTD: 25, PSS: 5</td>
</tr>
<tr>
<td>Sm</td>
<td>S</td>
<td>CREST: 7, DM: 12, PM: 96</td>
</tr>
<tr>
<td>RNP</td>
<td>S</td>
<td>SLE: 22, RA: 5, MCTD: 100</td>
</tr>
<tr>
<td>SSB</td>
<td>S</td>
<td>MCTD: 11, PSS: 8, SLE: 72</td>
</tr>
<tr>
<td>Centromere</td>
<td>C</td>
<td>SLE: 7</td>
</tr>
<tr>
<td>ScI-70</td>
<td>N + S</td>
<td>SLE: 23</td>
</tr>
<tr>
<td>Jo-1</td>
<td>Cyto</td>
<td></td>
</tr>
<tr>
<td>Nucleolar</td>
<td>N</td>
<td>SLE: 26, RA: 10, MCTD: 10, PSS: 10, CREST: 54</td>
</tr>
<tr>
<td>Histone***</td>
<td>H</td>
<td>DM: 43</td>
</tr>
<tr>
<td>PMSc</td>
<td>N - S/H</td>
<td>SLE: 46, RA: 10, MCTD: 5, PSS: 5, CREST: 5</td>
</tr>
<tr>
<td>PONA</td>
<td>CCD</td>
<td></td>
</tr>
<tr>
<td>Ribosomal</td>
<td>Cyto + N</td>
<td>SLE: 5, PM: 10</td>
</tr>
</tbody>
</table>

C - Centromere, CCD - Cell cycle dependent, Cyto - Cytoplasmic, H - Homogeneous, N - Nucleolar, R - Rim, S - Speckled
* - ANA - Anti-nuclear antibody  ** - Anti-ds DNA performed by Farr assay  *** - > 90% of patients with drug-induced SLE have these antibodies.
SLE - Systemic lupus erythematosus; RA - Rheumatoid arthritis; 1*SS - Primary Sjögren’s syndrome; MCTD - Mixed connective tissue disease; PSS - Progressive systemic systemic dermatitis; CREST - Calcinosis, Raynaud’s phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia
DM - Dermatomyositis; PM - Polymyositis

OTHER USEFUL TESTS

Lupus Anticoagulant and Anticardiolipin Antibodies

Lupus anticoagulant and anticardiolipin antibodies are closely related auto-antibodies that recognise similar phospholipid antigenic determinants. Lupus anticoagulant acts as an inhibitor of the prothrombinase complex which is required for conversion of prothrombin to thrombin. This inhibition prolongs in vitro clotting tests for both intrinsic and extrinsic pathways. Patients with these antibodies are artificially split into 2 groups:
1. Primary antiphospholipid syndrome: Antibodies plus clinical events without SLE and other related syndromes.
2. Secondary antiphospholipid syndrome: Antibodies plus clinical events with SLE or related syndromes.

There is very little evidence that the management of primary and secondary disease should be any different.

Current opinions as to the significance of these auto-antibodies are as follows:
1. Approximately one-third of patients with SLE have one or both antibodies. Patients with non-SLE disorders probably account for more than half of lupus anticoagulant and anticardiolipin antibody positive persons.
2. In patients with or without SLE or closely related disorders, a significant association exists between lupus anticoagulant, anticardiolipin antibodies and a history of thrombosis, neurologic disease and thrombocytopaenia.

3. Lupus anticoagulant and anticardiolipin antibodies have been detected in a low percentage (< 15%) of otherwise healthy women with a history of unexplained recurrent abortion. Aspirin has been shown to improve foetal outcome and thus all pregnant women with a history of these antibodies should be referred for specialist management, regardless of the presence of SLE.

4. Prospective studies of a series of patients with SLE show those who have lupus anticoagulant and anticardiolipin antibodies, and a history of multiple failed pregnancies, are at high risk of experiencing subsequent foetal loss. The prognostic value of these antibodies in patients without a history of foetal loss has only recently begun to be assessed.

HLA B27

There are many reported associations between certain HLA antigens, especially HLA B27, which is found in 8% of Caucasians, and a number of diseases. 50% of patients suffering from ankylosing spondylitis carry the HLA B27 antigen. Although the chance of HLA B27 positive individuals developing the disease is 100 times that of HLA B27-negative individuals, the vast majority of positive individuals will never develop ankylosing spondylitis. HLA B27 is also associated with more prolonged reactive arthritis and recurrent iritis. HLA B27 is associated with one of the variants of psoriatic arthropathy.

Complement

The interaction of antibodies with specific antigens to form immune complexes is a significant factor in the development of organ damage in rheumatic diseases. In excess, these complexes localise in blood vessels, activate complement pathways and cause inflammatory reactions. SLE patients with renal disease show consumption and glomerular deposition of early complement components of the classical pathways, hence reduction of C3, C4 and CH50 levels and increased levels of immune complexes. RA patients with systemic vasculitis may show a similar pattern.

Anti-Neutrophil Cytoplasmic Antibodies

Multiple patterns of anti-neutrophil cytoplasmic antibodies (ANCAs) are distinguishable by indirect immunofluorescence techniques using patient serum. Classical (c-ANCA) and perinuclear (p-ANCA) antibodies differ in their disease specificities and sensitivities. Atypical ANCs have many associations including sclerosing cholangitis, autoimmune liver disease and cystic fibrosis.

All patients with vasculitis diagnosed with Wegener’s granulomatosis (WG) (a variant of necrotising vasculitis) will have ANCA, and in greater than 95% it will be c-ANCA. However, to complicate matters, not all patients with WG have vasculitis, and are often termed limited stage disease. In some patients, this may progress to systemic disease, though in many the disease remains limited to the initial organ. During acute presentation with c-ANCA, antibodies are directed at the proteinase 3 antigen (Anti-PR3). The level of antibody is dependent upon disease activity, such that the degree of positivity falls to < 70% in patients with only limited disease, and to 30 – 40% in those patients who are in clinical remission. Presenting features include non-specific signs of systemic illness with evidence of respiratory tract involvement. The latter is a consistent feature of this particular illness, which may...
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Involve the entire respiratory tract from nose to lungs. Other manifestations of systemic disease are often present, particularly renal involvement presenting as a necrotising glomerulonephritis, inflammatory arthritis, and hearing loss. The disease can be rapidly progressive at any time, so early referral to a specialist is advisable. In some patients, fluctuating titres of c-ANCA may be related to the degree of disease activity and therefore may be useful in monitoring therapy. A rise in titre from a previous negative test almost always precedes a disease flare.

PR3 antibodies can be seen in bacterial endocarditis, possibly in association with renal failure. Immune suppression is not required and is contraindicated in this illness.

p-ANCAs are less specific than c-ANCAs. This is, in part, due to the fact that p-ANCAs are not a homogeneous group of antibodies. The major antibody involved in p-ANCA reactions is an antimi elastase and antilactoferrin antibodies, and these often give an atypical appearance. The atypical ANCA pattern is now used for the detection of auto-immune liver and biliary duct disease. Patients with classic Wegener’s granulomatosis rarely have p-ANCA.

Testing for ANCA by immunofluorescence and ELISA (proteinase 3 and myeloperoxidase) remains a valuable tool for the investigation of patients with suspected necrotising vasculitis. As with all auto-antibody investigations, the clinical picture needs to be taken into consideration when interpreting the results.

**Conclusion**

Despite the lack of definitive tests for the diagnosis of rheumatic and connective tissue diseases, there are many pointers to their diagnosis. Rheumatoid factor assays, although moderately sensitive for RA, lack specificity, being found in many illnesses and normal subjects, though the recent addition of CCP antibodies is a valuable tool. The ANA test remains a useful non-specific screening test for many connective tissue diseases. Elevated anti-ds DNA levels have a high association with SLE and its disease activity. Anti-ENAs are useful in identifying subsets of patients with connective tissue disease. In combination, the ANA Anti-ds DNA and anti-ENA tests are extremely helpful in the diagnosis of many of the connective tissue diseases. A negative ANA, anti-ds DNA and anti-ENA excludes the diagnosis of SLE. Tests for antihistone antibodies are useful in excluding drug-induced SLE. Tests for lupus anticoagulant and anticardiolipin antibodies identify patients with thrombosis and recurrent abortion. Presence of the HLA B27 antigen is important, but not essential, in the diagnosis of ankylosing spondylitis. Immune complexes and serum complement levels are useful in the monitoring of subsets of SLE and RA patients with vasculitis. Testing for ANCA is useful in the investigation of patients with suggested necrotising vasculitis.