LABORATORY STANDARDS IN DIAGNOSIS AND THERAPY MONITORING OF RHEUMATOID ARTHRITIS

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6.1 Introduction

Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease with a prevalence of 0.5% to 1% in the general population and a male to female ratio of 2.5:1. The disease usually manifests at the age of 40-70 years, its incidence rising with age. The lowest prevalence of RA is reported from rural Africa, and highest in particular groups of native Americans (Pima and Chippewa). Mainly, it is an inflammation of the synovial membrane (which lines the joint cavity and secretes synovial fluid acting as a 'lubricant') and hyperplasia of the synovial tissue with considerable infiltration of lymphocytes, macrophages and plasma cells. Typical clinical symptoms of RA include symmetrical synovitis, which initially involves only one pair of small finger joints (proximal interphalangeal, metacarpophalangeal or metatarsophalangeal joints) with painful episodes and erythema. During the disease characterized by acute inflammatory episodes, destructive changes of the joints develop and result in deformity and progressive functional impairment. RA is also a systemic inflammatory disorder with extra-articular manifestations such as rheumatoid nodes in subcutaneous tissues, pleuritis, pericarditis, ulcerations, and digital gangrene due to immune complex deposits.

6.2 The pathophysiology of RA

Genetic studies have pointed to the association of RA with HLA-DRB 10404 and DRB 10401 alleles of the MHC II complex. The basic function of HLA class II molecules is presentation of peptide antigens to CD4+T lymphocytes, which predominate in the synovial membrane infiltrates of RA patients, suggesting that RA is induced by an as yet unidentified arthritogenic antigen. This antigen may be of an exogenous (viral protein) or endogenous (citrullinated peptides) origin. The antigen activated CD4+ T cells stimulate monocytes, macrophages and synovial fibroblasts for the production of cytokines (IL-1, IL-6, TNFα), secretion of matrix metalloproteinases mediated by surface receptors (CD69 and CD11) and release of soluble mediators (IFNγ, IL-17). They also stimulate B lymphocytes by surface contact for immunoglobulin production, including rheumatoid factor (RF). Furthermore, activated CD4+ T cells express on their surface osteoprotegerin ligands, which stimulate osteoclastogenesis. Among the cytokines released by activated mononuclear cells, TNFα and IL-1 have been postulated to play a primary role in the pathogenesis of RA, based on their high concentrations in serum and synovial fluid of RA patients. They also are potent stimulators of mesenchymal cells such as synovial fibroblasts, osteoclasts and chondrocytes,

stimulating their release of metalloproteinases that act destructively on the surrounding tissue. Furthermore, TNFα and IL-1 inhibit the production of tissue metalloproteinase inhibitors by synovial fibroblasts. By the induction of IL-11 release, TNFα may stimulate osteoclastogenesis, which then leads to bone degradation.
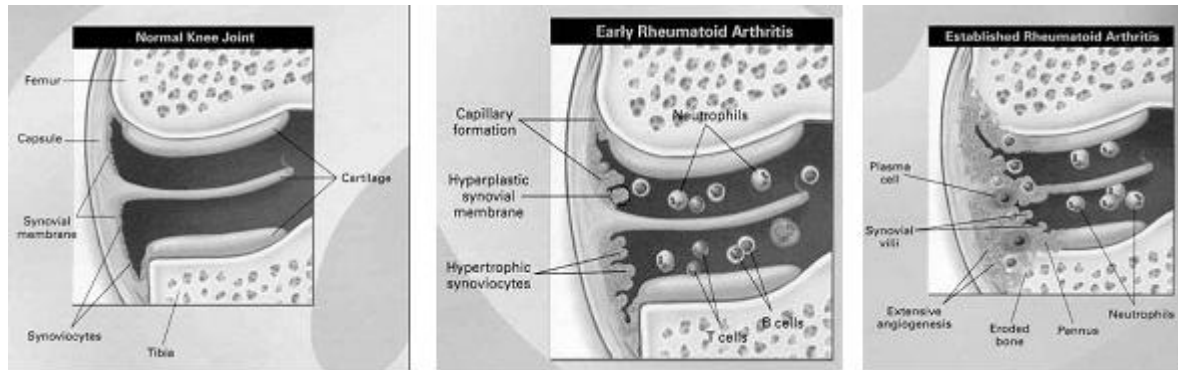
6.3 Articular lesions in RA

Figure 1. Schematic presentation of normal knee joint (a); joint in early RA (b); joint in established RA (c). (From: Choy EHS, Panayi GS. Mechanisms of disease: cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 2001;344:907-16)

In a normal joint (Figure 1a) synovia consists of synovial membrane (1 or 2 cell layers) and a lower layer of loose connective tissue. The cells lining the synovia are known as A synoviocytes (macrophage-like synoviocytes) and B synoviocytes (fibroblast-like synoviocytes). In the early stage of RA (Figure 1b), hyperplasia of the synovial membrane (a layer of 10 or more cells in thickness) occurs. The connective tissue layer underlying the membrane is exposed to massive infiltration with mononuclear cells (T and B lymphocytes, macrophages and plasma cells), which stimulate, by their cytokine release, the expression of adhesion molecules on the endothelial cells of the synovial vasculature. This, in turn, results in an increased neutrophil infiltration.

Furthermore, they activate angiogenesis, which additionally contributes to this effect. Neutrophils release elastases and proteases, which degrade proteoglycans in the surface layer of the cartilage. Proteoglycan depletion leads to immune complex precipitation in the surface layer of collagen and exposure of chondrocytes. Stimulated by TNFα and IL-1, chondrocytes and synovial fibroblasts release destructive metalloproteinases, primarily stromelizine and collagenases as well as catepsins. The formation of such a locally invasive synovial tissue, so-called pannus, is a characteristic histologic lesion in established RA (Figure 1c). Initially, the cartilage penetration with synovial pannus consisting of mononuclear cells and fibroblasts occurs. This cellular pannus is presumed to mostly originate from B synoviocytes, in which the lack of contact inhibition of proliferation has been demonstrated by in vitro assays. This feature is consistent with the transformed cell phenotype. In the later stage of disease, cellular pannus is being replaced by fibrous pannus, which consists of a minimally vascularized layer of pannus cells and cartilage covered with a layer of collagen.

6.4 The diagnosis of RA

RA is diagnosed primarily according to clinical manifestations of the disease, whereas serologic support has been restricted to the determination of IgM rheumatoid factor (IgM-RF), which is characterized by limited sensitivity and specificity for RA. Recently, laboratory diagnosis has been improved by the detection of antibodies to cyclic citrullinated peptides (anti-CCP).

The diagnosis of RA is based on the revised (1987) classification criteria set by the American College of Rheumatology (ACR) (Table 1).

Table 1. The ACR 1987 revised criteria for the classification of rheumatoid arthritis

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP*, MCP*, wrist, elbow, knee, ankle, and MTP* joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, on extensor surfaces, or in juxta-articular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

PIPs = proximal interphalangeal joints, MCPs = metacarpophalangeal joints, MTPs = metatarsophalangeal joints

The diagnosis is confirmed if 4 of these 7 criteria are present and persist for at least 6 weeks. Unfortunately, the ACR criteria are unsuitable for early diagnosis, which is crucial because irreversible damage of the joints with destruction of the cartilage begins at this stage of the disease. Therefore, the European League of Arthritis and Rheumatism (EULAR) recommends findings of more than 3 swollen joints, tenderness at metatarso- or metacarpophalangeal joints, and morning stiffness of more than 30 min for a well-founded suspicion of RA.

Modern treatment of RA is being shifted towards aggressive antirheumatic therapy at an early stage of the disease because irreversible joint damage develops within the first two years. Because the potentially toxic second-line drugs can cause serious side effects, a highly specific diagnostic test that would identify patients at high risk of a destructive form of disease would be desirable.

6.5 Autoantibodies in the diagnosis of RA

6.5.1 Rheumatoid factor

Rheumatoid factor (RF) is an antibody to antigen determinants of the IgG Fc fragment. The most common isotypic profile in RA is the concomitant presence of IgM and IgA, or of IgM, IgG and IgA antibodies. In clinical practice, only IgM antibodies (Figure 2) are generally determined by the methods based on RF reaction with Fc fraction of human or animal (rabbit) antibody. Agglutination tests such as Waaler-Rose test with rabbit IgG coated sheep erythrocytes, or latex test with human IgG coated latex particles can be qualitatively or semiquantitatively (titration) performed, whereas immunonephelometry and ELISA are used for quantitative measurements.
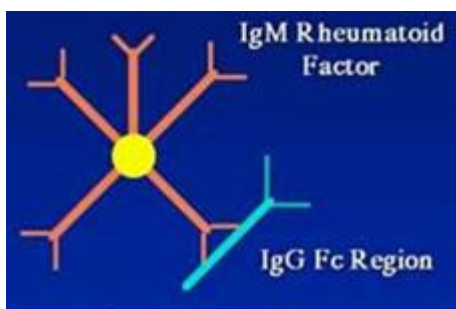

Figure 2. Schematic presentation of IgM-rheumatoid factor

The potential physiologic role of IgM RF includes the following:
1) the IgM bound on the surface of B lymphocytes enhances antigen presenting efficiency of these cells for the antigen complexed with IgG; and
2) secreted IgM - stabilizes low-affinity IgG-Ag complexes
   a. removes immunocomplexes via complement activation
   b. improves opsonization

The specificity, sensitivity and predictive values of RF as an RA marker suffer from some limitations. So, RF is positive in 60% to 80% of RA patients, indicating that a negative result does not rule out the diagnosis of RA. Furthermore, RF is positive in other rheumatic (Sjögren's syndrome, MCTD, systemic lupus erythematosus) and nonrheumatic diseases (mixed cryoglobulinemia type II - monoclonal IgM RF, chronic liver diseases, subacute bacterial endocarditis, other bacterial, viral and parasitic infections). RF is positive in up to 5% of healthy individuals under age 50, and in up to 25% of those older than 70 years. Thus, positive RF as an isolated finding without clinical criteria has no diagnostic relevance. However, RF determination is useful in the differential diagnosis of rheumatic diseases, and as a prognostic factor because its high titer is associated with rapid progressive articular destruction and extra-articular manifestations (subcutaneous rheumatic nodes,

polyneuropathies, vasculitis, etc.). RF specificity for RA increases with positive RF finding on two consecutive determinations, increased RF titer, reactivity with rabbit and human IgG (positive Waaler-Rose and latex test results), and distribution among IgM, IgA and IgG classes. Although some studies have shown a high IgG RF titer to be a risk factor for the development of vasculitis, while IgA correlates with bone erosions, differentiation of RF isotypes does not contribute significantly to the prognostic value of RF determination.

6.5.2 Antibodies to cyclic citrullinated peptides (anti-CCP)

In 1964, Nienhuis and Mandema described an antibody targeted to a component of the keratohyalin granules surrounding the nucleus of the buccal mucosa (BM) cells, yielding characteristic perinuclear fluorescence. Therefore, they named this antibody antiperinuclear factor (APF). Studies have shown the presence of APF in 49% to 91% of RA patients, with a specificity ranging between 73% and 99%. However, in spite of its high specificity, APF test has never been widely accepted because of the substrate inappropriateness for IIF. Namely, a high percentage of APF positive BM cells can only be found in some 5% of healthy donors. In 1979, antikeratin antibodies (AKA) were described and their presence demonstrated by IIF on cryostatic sections of rat esophagus. The target antigen was present in the cornified layer of the stratum corneum. The sensitivity of AKA test is 36% to 59%, and specificity 88% to 99%.

Two arguments have supported the concept that AKA and APF are directed against immunologically related antigens: (a) the antigens are localized in similar types of squamous epithelium; and (b) high correlation for the presence of AKA and APF in serum of RA patients. Indeed, it has been demonstrated that the antigen recognized by AKA and APF is filaggrin (filament-aggregating protein), a protein synthesized during terminal differentiation of mammalian epithelial cells and involved in the organization of cytoskeletal structure. Therefore, AKA and APF antibodies are more correctly referred to as "antifilaggrin" (AFA) antibodies. Synthesized as a highly phosphorylated precursor protein named profilaggrin, deposited in granules, filaggrin is being released via proteolytic cleavage during cell differentiation. In this stage about 20% of arginine residues are deiminated to citrulline by the action of the peptidylarginine deiminase enzyme (PAD). In 1998, Schellekens et al. demonstrated citrulline to be a major constituent of the antigenic determinants recognized by AFA antibodies. There are at least 5 subtypes of PAD enzyme expressed in a cell type/tissue specific manner. Therefore, expression of this ubiquitous modifying enzyme in synovial cells as well as in hematopoietic cells infiltrating synovia during articular inflammation is not unexpected. The mechanism of the autoantigenic epitope formation probably involves the development of immune tolerance to the nonmodified protein. On cell differentiation, arginine residues are modified into citrulline and, if presented to the immune system (e.g., on massive cell damage or uncontrolled apoptosis), may induce immune response. However, filaggrin expression is restricted exclusively to squamous epithelium, and is not expected in synovial cells. Recent studies suggest that PAD enzyme most likely causes local citrullination of synovial proteins like fibrin, histones and vimentin. Besides AKA and APF, another RA specific antibody, anti-Sa antibody, was also found to be targeted to citrullinated protein, in this case vimentin, a cytoskeletal intermediate filament protein found in mesenchymal cells. Immunoblotting assays and ELISA using filaggrin purified from human epidermis as an antigen have been

developed for the detection of AFA. Unfortunately, the sensitivity of the assays was poor, mainly because of the heterogeneity in the filaggrin amino acid sequence, charge and degree of deimination. These problems were overcome by the use of linear synthetic citrullinated peptides as substrates in ELISA. In further attempts to improve the sensitivity, cyclic variants of peptides in which the citrulline residue was optimally exposed to antibody binding were used. This led to the first generation of the anti-cyclic citrullinated peptide test (anti-CCP1). Further investigations in which RA sera were used to select the most reactive species from libraries of citrullinated peptides resulted in second generation of anti-CCP assay (anti-CCP2). Recent studies have confirmed that the CCP2 test equals the RF level of sensitivity (60%-80%), however, coupled with a much higher specificity (95%-98% vs. 70%-80%). In RA patients with high disease activity or severe joint damage the sensitivity of CCP2 approached 81%-84%. Anti-CCP test proved to be a powerful diagnostic tool, especially in ambiguous cases or RF negative patients with RA, or to discriminate RA patients from those with other RF positive cases. Also, it appears to be a good prognostic marker since it can be detected very early in the course of the disease and has a high discriminating power between erosive and non-erosive RA. According to recent clinical studies, RA patients with positive baseline anti-CCP develop significantly more radiological damage than anti-CCP negative patients.

6.6 Biological markers for the management of RA

To assess the disease severity, a number of standardized rating systems according to pain and mobility indices are in use. Laboratory monitoring of RA patients is still limited mainly to inflammation parameters such as CRP and ESR, which are not joint specific and are poorly correlated with cartilage damage. Radiography of the hands and feet is still a gold standard for assessing joint damage in RA, although it allows neither early detection of joint tissue damage nor efficient monitoring of treatment. Specific and sensitive biochemical markers reflecting abnormalities in the metabolism of joint cartilage could have a major role in the detection of early damage and thus in identifying patients at a high risk of progressive type of RA, who are candidates for disease-modifying anti-rheumatic drug (DMARD) therapy. Also, such markers could be useful for monitoring drug efficacy. Besides the promising role of anti-CCP, some cartilage turnover biomarkers could serve for the same purpose. Articular cartilage is avascular, non-innervated hyalin cartilage tissue in which chondrocytes are located in lacunae of the extracellular matrix. Cartilage matrix contains water (up to 70%), collagen fibers, proteoglycans, non-collagenous matrix proteins and lipids. The principal structural component of cartilage is an extensive network of collagen molecules arranged in fibrils. A large aggregating proteoglycan, aggrecan, resides within the fibrillar network. The predominant collagen in the extracellular matrix of articular cartilage is type II collagen. Like collagen type I, it is synthesized as a pro-form, with propeptide extensions at both N-terminus (PIINP) and C-terminus (PIICP). Measurement of circulating levels of these propeptides can be used as a marker of collagen type II synthesis. Mature collagen type II consists of a triple helical structure with short telopeptides at both ends. The telopeptides are covalently cross-linked to other collagen strands forming a rigid fibrillar network. Cleavage of type II collagen triple helix by collagenases results in the generation of neoepitopes at the cleavage sites. The final cleavage products are released from the cartilage tissue and can be detected by immunoassays in body fluids (synovial fluid/serum/urine).

Besides type II collagen, the second major component of the articular cartilage extracellular matrix is the proteoglycan aggrecan. It is composed of a protein (core protein) and glycosaminoglycan (GAG) chains that are covalently attached to the core protein. Fragments of the aggrecan molecule released to body fluids can also serve as markers of cartilage turnover. Finally, several noncollagenous proteins, including cartilage oligomeric matrix protein (COMP) and human cartilage glycoprotein-39, also called YKL-40, are synthesized by both chondrocytes and synovial cells, and therefore serve as markers of upregulated cartilage turnover. A summary of cartilage turnover biomarkers and their possible clinical utility in RA (based on published clinical studies) is presented in Tables 2 and 3.

Table 2. Biomarkers of cartilage degradation

Biomarker	Description	Specimen	Utility in rheumatoid arthritis
CTX-II	6 amino acid epitope at C-telopeptide fragment of type II collagen	Urine	• Increased levels are an indication of increased cartilage degradation. • Levels correlate with the extent of joint destruction. • Baseline levels can predict an increased risk of radiologic progression in patients with early RA. Higher CTX-II levels can indicate a higher risk of progression. • Can be used for monitoring the effect of anti-rheumatic drug therapy.
C2C	Epitope at C-terminus of the 3/4 length type II collagen cleavage product of collagenase action	Serum, synovial fluid	• Increased levels are an indication of increased cartilage degradation. • High levels predict rapid progression of joint damage following early diagnosis. • Levels reflect efficacy of anti-rheumatic drug therapy.
C1,2C	This epitope results from secondary cleavage of the long C2C collagen II (also type I collagen) cleavage product	Serum, synovial fluid	• Increased levels are an indication of increased cartilage degradation. • High levels predict rapid progression of joint damage following early diagnosis.
COMP	Protein of trombospondin gene family, synthesized by chondrocytes and synovial cells activated by proinflammatory	Serum, synovial fluid	• Increased serum COMP indicates cartilage degradation and can rule out nonspecific inflammatory processes and less cartilage-destructive forms of arthritis. • COMP levels can be used for monitoring the effect of anti-

	cytokines		• rheumatic drug therapy. • Prognostic factor in early RA. Elevated serum levels indicate an unfavorable prognosis of rapid joint destruction.

Table 3. Biomarkers of cartilage synthesis

Biomarker	Description	Specimen	Utility in rheumatoid arthtritis
CPII	Type II collagen C-terminal propeptide	Serum	• CPII measurement can distinguish OA from RA patients. Serum CPII levels are suppressed in OA (but elevated in OA cartilage) and significantly elevated in rapid progressive and chronic RA. However, baseline levels were not predictive of rapid progression of radiologic damage.
CS846	Epitope on the chondroitin sulfate chains of aggrecan	Serum	• CS486 levels can distinguish between rapid progressive RA and slow chronic disease. • Elevated serum levels of CS486 indicate a more favorable prognosis of slow joint destruction. • Levels reflect efficacy of drug therapy.
YKL-40	Glycoprotein synthesized by chondrocytes and synovial cells activated by proinflammatory cytokines. It is also found in the liver. Levels highly correlate with CRP and ESR suggesting that it mainly reflects synovial inflammation	Serum, synovial fluid	• Levels of YKL-40 in synovial fluid are 15-fold those in serum. Synovial YKL-40 levels reflect the degree of synovial inflammation in RA. • YKL40 serum levels reflect RA disease activity and response to anti-rheumatic drug therapy. • YKL-40 serum levels are related to progression of joint destruction in early RA.

Because of their rapid, dynamic changes, biochemical markers of upregulated cartilage turnover should be valuable for clinical development of new drugs to decrease the progression of joint damage. However, the use of these biomarkers is still limited by insufficient knowledge of the metabolism and clearance of these molecules as well as of the contribution of nonarticular tissues to their SF, serum and urine concentration. Extensive human clinical studies are therefore required to establish the true clinical value of these biomarkers.

Literature

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