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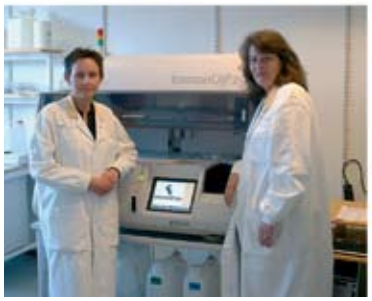

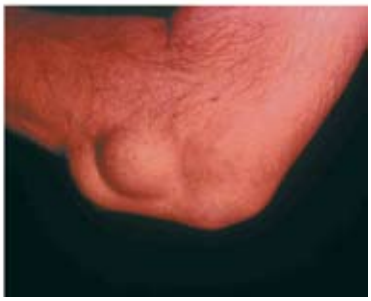

EliA™
Excellence in Autoimmunity

CONTENTS

Editorial

Automation is nothing new in clinical laboratories: the speed, quality, and diversity of instruments designed to perform testing on samples have continuously improved since the second half of last century. These instruments first mimicked manual methods but later took advantage of newer technologies. First clinical chemistry and then haematology were impacted by these instruments, which allowed laboratories to meet the large increase in testing demand without adding greatly to the number of staff and costs. However, the autoimmunity laboratory has been one of the latest members of clinical laboratories to incorporate automation. Mr. José Manuel González-Buitrago from Salamanca, Spain, has written an overview article on automation for us with all aspects specific for the autoimmunity laboratory (see page 3)

For autoimmune diagnostics, particularly in big labs with a high sample throughput, automated MTP systems are most frequently used. For more than two years now, Varelisa and DSX have worked as a team in MTP automation. Mr. Warren Taylor uses this combination in his routine and gave a short interview to our colleague from the UK about the automation in his lab (see page 4).

More and more laboratories would prefer fully automated, closed systems with a maximum of time-saving, but until last autumn, this possibility for autoimmunity had often been announced by commercial companies but had never come to the market. With EliA on ImmunoCAP™250 the first fully automated closed automate was introduced in November 2005. The throughput is lower than a high throughput MTP system but ImmunoCAP™250 offers a flexibility which is unique in autoimmunity (see article on page 5).

With the introduction of EliA CCP the testing of anti-CCP antibodies has been automated for the first time, worldwide. In the few years since its first description, this disease marker for rheumatoid arthritis has been accepted by autoimmunity laboratories at an exceptional rate. Although not reimbursed in most European countries, most rheumatologists are aware of its clinical significance and its enormous diagnostic usefulness. On page 6, Elke Meier gives a short overview on the clinical background of rheumatoid arthritis and this is followed on page 7 by an article by Carsten Konrad from Dresden, Germany, who goes into more detail on the serodiagnosis of rheumatoid arthritis.

Pleasant reading

EliA Journal *is the Journal of*

Phadia

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Automation in the Autoimmune Laboratory

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Currently, most of the work in clinical laboratories is performed using automatic analysers that combine sample processing and the measurement of the signals generated with a data presentation of the final results. The autoimmunity laboratory has been one of the latest members of clinical laboratories to incorporate automation. One of the main reasons for this has been the strong implantation of indirect immunofluorescence (IIF) as a basic technique in autoimmunity studies. Immunofluorescence microscopy is difficult to automate because it is a technique that essentially depends on the subjective interpretations of observers. Nevertheless, the constant and increasing demand for tests as a result of the detection of new autoantibodies, together with the demonstration of their clinical usefulness, has pushed autoimmunity laboratories to advance in recent years (1). For the above reasons, such laboratories are currently on the way to full automation.

For years, indirect immunofluorescence has been a basic technique in autoimmunity laboratories. Autoantibodies produce characteristic fluorescence images called fluorescence patterns. One of the objections to IIF is that the technique cannot be completely automated. Over the past few years, robotic systems for microplate management have incorporated the ability to process slides with cell or tissue preparations. These systems add serum samples, incubate, wash, and add fluorescent conjugates automatically. Thus, the slides are already prepared for microscopic observation. Recently, in attempts to automate the stage of microscopic observation of IIF, a computer-assisted system for the classification of interphase HEp-2 cell immunofluorescence patterns for autoimmune diagnoses has been described (2). By use of a software package based on image analysis, feature extraction, and machine learning algorithms, relevant characteristics describing such patterns can be detected. The system is claimed to be of use in discriminating between positive and negative sera for a previous classification of the most important patterns. However, we believe that this system is not a likely candidate for routine use in the autoimmunity laboratory owing to its low-resolution capacity.

In considering IIF automation, it is also important to note that in recent years IIF has been questioned as an essential procedure in autoimmunity laboratories, at least as far as the most requested test - antinuclear antibodies (ANA) - is concerned. As pointed out by Nossent (3) "given the fact that a positive ANA by IIF always needs further workup to determine antigen specificity and the fact that a negative ANA by IIF is only useful in excluding the relatively rare disorder systemic lupus erythematosus (SLE), it may be time to consider alternatives to ANA-IIF testing". We agree with Nossent that it is now time for specific autoantibody testing and for allocating the ANA-IIF test to its well-deserved place in medical history (3).

The number of autoantibodies that autoimmunity laboratories analyse and measure has increased spectacularly. Currently, immunoassay methods occupy a central position among the techniques used in this type of laboratory. The main characteristic of autoimmunity laboratories, and indeed the one that differentiates them from other laboratories that use immunoassays as basic techniques, is that they determine antibodies (autoantibodies) and not antigens. For this reason, immunoassay techniques must employ antigens as reagents.

Immunoassays can be performed either without or with labelled reagents. In non-labelled immunoassays, antigen molecules react with antibody molecules to form immune aggregates that can be detected by their turbidity. To obtain greater sensitivity, the antigen in the reagent (in the case of autoantibody measurements) can be attached to a solid particle. In the autoimmunity laboratory a typical parameter measured by turbidimetry or nephelometry using the technique described in biochemical automatic analysers is Rheumatoid Factor (RF).

Reagent-labelled immunoassays use molecules called labels or tracers which are attached to the antigen reagent in autoantibody measurements, to show that an immune reaction has occurred. The labels used are radioactive isotopes, enzymes, fluorescent compounds, and luminescent compounds. This kind of immunoassay consists of two stages; the first stage is the immune reaction between antigen and antibody, while the second stage is the measurement of the label.

Today, labelled immunoassays offer a basic technique in clinical and autoimmunity laboratories. The most frequently used labels are enzymes and fluorescent compounds. Two of the main components of a labelled immunoassay for autoantibodies are the solid phase and the antigen. Microplates, beads, and magnetic particles are the solid phases employed in labelled immunoassays. Microplates are the solid supports most widely used in autoimmunity testing. As regards antigens, the specificity of a labelled immunoassay for autoantibody measurement is strongly dependent on the quality of the antigen used. Commercial reagents for autoantibody determinations use one of the following approaches as the antigen bound to the solid phase: cell extracts; antigens purified from animal tissues; recombinant antigens; mixtures of cell extracts and recombinant antigens, and mixtures of purified and recombinant antigens.

Automation in the measurement of autoantibodies requires a tandem of components: reagents and apparatus. When the reagent/apparatus pair is considered within the context of the automation of heterogeneous immunoassays (those that require separation of bound and non-bound fractions of immune reactions), three possibilities may be considered:

1. Open systems

These systems can be considered as semi-automated. In general, they are workstations that use microplates as a support for the reactions. They basically consist of three components: a pipetting module for sample and reagent dispensation, a washing module, and a reading and data-processing module. These systems allow the use of reagents provided by different suppliers.

2. Microplate processors

These are computer-controlled Cartesian robots that perform all of the operations with the samples and reagents needed for a heterogeneous immunoassay to be carried out, including the reading of the signal produced. As in open systems, microplate processors allow the use of reagents provided by different suppliers.

3. Integrated systems

These are compact analysers that include all the components required to perform an immunoassay in a single device. They use reagents that are specifically designed for them and are provided by the manufacturer of the analyser, and they do not allow the use of other reagents.

At present, the market offers several microplate processors for the measurement of autoantibodies. Some of these processors combine the performance of immunoassays for autoantibodies with the preparation of plates for indirect immunofluorescence. Different reagent manufacturers commercialise kits for autoantibody measurements on microplate processors. Phadia Varelisa offers a set of reagents for autoantibody measurements using microplate strips. Varelisa uses high quality antigens from a recombinant source. Ninety percent of Varelisa tests are based on recombinant antigens, most of them produced in eukaryotic cells (insect cell/baculovirus expression system). Varelisa reagents are readily adapted to different commercial microplate processors, with excellent results.

Recently, a microsphere-based immunoassay for running in microplate wells with flow cytometry readings has been developed for the simultaneous determination of several autoantibodies (multiplexing technology). Theoretically, up to 100 autoantibodies can be measured simultaneously. Even so, they are not totally automated systems because after the immunoassay has been carried out the microplates must be manually carried to the flow cytometer for reading of the signals produced. Currently, several commercial kits have been introduced for antinuclear antibody specificities (4-6). Thus, it appears that particle-based flow cytometric assays of autoantibodies have considerable potential, although today they are not used very much in autoimmunity laboratories.

Few automated immunoassay analysers have reagents dedicated to antibody measurements. Some years ago, Roche developed reagents for autoantibody measurements on their automated Cobas Core analyser (7,8), but this device has been withdrawn from the market. One system on the market is ImmunoCAP from Phadia. For autoimmunity, there are two commercialised models: ImmunoCAP100 and ImmunoCAP250. The ImmunoCAP systems use EliA reagents. They apply the principles of microplate coating to single polystyrene wells, which are automatically dispensed and processed in ImmunoCAP instruments. The two ImmunoCAP models are designed for low and high laboratory workloads, respectively. We have previously evaluated EliA reagents in ImmunoCAP systems, with excellent results (9,10).

To conclude, autoimmunity testing has come of age with the constant and increasing detection of new autoantibodies and the demonstration of their clinical usefulness. This increase in the workload has guided autoimmunity laboratories on the road to automation. ■

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Varelisa on an Automated MTP Processor in Routine

EliA Journal recently interviewed Mr Warren Taylor, a Senior Biomedical Scientist working in Immunology at the Churchill Hospital Oxford.

EliA Journal: Could you describe the laboratory for us ?

WT: We are a Regional Immunology laboratory performing a large number and range of diagnostic tests for our Trust and local GPs. In addition, we provide a specialist Immunology diagnostic service to local and other UK hospitals. This specialist service includes tests not offered by many other laboratories in the UK, for example, Neuroimmunology and Immunodeficiency.

EJ: When did you decide you needed some ELISA Automation?

WT: We had the usual issues in laboratories of recruitment and retention as well as an increasing test workload. We started looking at automation 7 years ago when we were offered a 2 plate system that had integrated plate washing and reading. This was one of the first systems available and unfortunately it didn't live up to our expectation. It did not have the capacity to deal with the rapid increase in testing we were experiencing and was not flexible enough to be used to automate some of our specialist in-house ELISAs. There were some reliability issues as well which limited the instrument's usefulness.

EJ: How did you resolve the issues?

WT: We looked around for an alternative Instrument and found the Dynex Technologies DSX Instrument. This is a 4 plate system with a large sample and reagent capacity. The instrument was just what we needed to take the strain of performing our routine ELISA work.

EJ: What do you like about the Dynex DSX?

WT: We like the flexibility the instrument provides. We can run small batches of samples for a number of different analytes or we can run large batches of samples for a single analyte. The ability to continually reload the instrument during running with new samples and ELISAs is essential as our workload is so high. The ability of the instrument to use a range of different sample tubes is very important as we receive a wide range of sample tubes from the hospitals referring samples to us. In addition, we have been able to automate difficult in-house assays measuring antibodies to microbial antigens. This has considerably reduced the number of time-consuming manual ELISAs we have to run.

EJ: What difference does having the DSX make to your day?

WT: We now have a reliable and flexible automated system able to run the majority of our ELISA assays. I am now able to find time for the more interesting aspects of running an Immunology laboratory.

EJ: Would you be able to manage your laboratory without the DSX?

WT: No

EliA™ on ImmunoCAP™250

The Future in Autoimmunity Testing - now!

Nina Olschowka

Phadia GmbH, Munzinger Straße 7, D-79111 Freiburg, Germany

Time for the essentials

ImmunoCAP™250 is a highly automated system for autoimmunity (EliA™) and allergy (ImmunoCAP™) testing. Designed to assist laboratory workflow by reducing hands on time, it processes samples from test request to final result with minimal intervention.
Barcode identification of all reagents, mainframe worklist downloads or bacode/manual sample identification is combined with on-board dilution to reduce manual handling and minimise errors.
All reagents can be stored on board to minimise loading times. User-friendly operation makes test handling and data processing simple and convenient.

Cost efficient and flexible

The modular EliA™ system allows for patient-specific, economical routine use. One standard curve is used for all analytes within a method and calibration is required only monthly.
For every sample, an individual test panel can be compiled. Single samples can be run immediately and prioritised if the results are needed urgently and reflex testing from EliA™ Symphony to specific ANA analytes is possible.

Intuitive, user-friendly handling

The unique ImmunoCAP™ Information Data Manager (IDM) software allows the integration of several ImmunoCAP™ Instruments (including ImmunoCAP™100 and ImmunoCAP™1000) into one network.
IDM manages all results, an in-depth quality control programme as well as the total laboratory stock. Additionally, IDM has a high-capacity patient database and allows detailed documentation of results.
On the instrument itself, logical software accessed via a built-in touch screen, makes the handling of the system very easy.

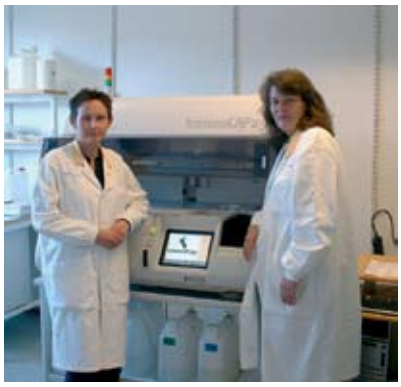
Maximum specificity and sensitivity in autoimmunity testing

To ensure the highest quality of our autoantibody tests, it is crucial to use very pure, conformationally-correct antigens. We use human recombinant proteins and synthetic peptides whenever it increases antigenicity and thus sensitivity and specificity.

EliA has been specifically designed to offer clinically relevant results in autoimmunity testing while meeting the laboratory demands of efficient and cost-effective workflow. ■

Customer Experience

"At the Immunological Laboratory in Gothenburg, Sweden, we have had the privilige to test EliA on the ImmunoCAP250 during a week last autumn.
We have previously run Allergy on the instrument and we think that loading and analysing EliA was as smooth as it is for Allergy. We also got the opportunity to load both allergy and different EliA analytes at the same time. This worked out excellently. We consider the ImmunoCAP250 to be a user friendly, logical and safe instrument."



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The Clinical Picture of Rheumatoid Arthritis

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Rheumatoid Arthritis (RA) is the most common form of inflammatory joint disease and is found in 0.8 – 1 % of the general population. In the USA, 2.1 million people are affected, and worldwide there are 165 million RA patients. It represents a chronic, systemic autoimmune disease in which the immune system attacks the individual's own tissue. It is characterised by symmetric, erosive joint synovitis. The Diagnosis of RA is basically clinical. Serological evidence, however, may have an additional supportive value.

Onset

The onset of Rheumatoid Arthritis is occasionally (in 5 – 8 % of all cases) sudden, occurring over 24 to 48 hours. More typically, however, RA develops more insidiously over a period of several weeks (55 – 70 % of all cases), and 15 – 20 % of patients have an intermediate onset. The initial symptoms may be systemic or articular. In some patients, fatigue, malaise or diffuse musculoskeletal pain may be the first symptoms. Often the patient first notices morning stiffness in one or more joints, usually accompanied by swelling, pain on movement and tenderness in the joint. This may be associated with local heat, but not erythema. Morning stiffness is probably due to accumulation of oedema fluid during sleep. The number of affected joints is highly variable, but almost always the process is eventually polyarticular. Due to the insidious onset and the unspecificity of first symptoms, the mean delay from disease onset to diagnosis is 9 months.

Key clinical features

Key clinical features of RA are symmetric polyarthritis with morning stiffness and subcutaneous nodules that may develop during later stages of disease in about 20 – 35 % of patients. Rheumatoid nodules consist of a central area of necrosis that is surrounded by a layer of fibroblasts and a collageneous capsule. In most cases they are found over bony areas of pressure, such as elbow, finger joints or ischial and sacral areas. Rheumatoid factor (RF) is almost always found in the serum of patients with rheumatoid nodules.

In the typical case, inflammatory polyarthritis is bilateral and symmetric involving small and large joints both in the upper and lower extremities with sparing of the axial skeleton except the cervical spine. In most patients, small proximal joints of fingers and toes as well as ankles or wrists are the first to be affected. Typical is a "spindled" appearance of the fingers that is caused by swelling of the proximal and MCP (metacarpophalangeal) joints, but not of the distal joints. Knees (80 % of patients), elbows, shoulders (60 % of patients), and the cervical spine (60 – 70 % of patients) also become involved as the disease progresses. Symmetric polyarthritis may lead to radiographically detectable changes. Characteristic radiographic features of RA are bone erosion, cartilage loss, juxta-articular osteopenia and soft tissue swelling. However, early in the disease process, these may not yet be evident. Later in the course of disease radiographs may show joint space narrowing, diffuse osteoporosis, and eventually marginal bone erosions. The destructive changes occurring in joints affected by RA include degeneration of cartilage, bony erosions, and destruction of ligaments and tendons. A further element is pannus, an expanding mass of synovial lining cells that extends into articular cartilage and bone and which is filled with inflammatory cells.

The main clinical features of RA are summarised in the criteria of the American College of Rheumatology (ACR) for classification of RA that were revised in 1987 (Table 1).

The ACR criteria that were developed as a classification tool are often used as aids in diagnosis. However, it has been shown that in their current form they are not really suitable for diagnosis of RA in early phases as they mainly reflect the features of the disease in its chronic stage. Early diagnosis of RA, however, is of particular importance, because on the one hand patients in the beginning of the disease often present with atypical symptoms and on the other hand approximately 90 % of RA patients already have radiological evidence of damage by the end of two years.

Inflammatory activity in RA patients can be determined by measurement of ESR (erythrocyte sedimentation rate) or CRP (C-reactive protein). Rheumatoid factor (RF) and anti-CCP

Patients are classified as having RA if they satisfy at least four of the seven criteria

Criterion	Definition
Morning stiffness[1)]	In and around joints, lasting for at least one hour
Arthritis of three or more joint areas[1)]	Observed by a physician simultaneously, with soft tissue swelling or joint effusions, not just bony overgrowth. The 14 possible joint areas involved are right or left proximal interphalangeal (middle and basal joints of fingers and toes), metacarpophalangeal (knuckle-bones) , wrist, elbow, knee, ankle, and metatarsophalangeal (midfoot) joints.
Arthritis of hand joints[1)]	Arthritis of wrist, metacarpophalangeal joint or proximal interphalangeal joint
Symmetric arthritis[1)]	Simultaneous involvement of the same joint areas on both sides of the body
Rheumatoid nodules	Subcutaneous nodules over bony prominences, extensor (muscle) surfaces or juxta-articular (near the joint) regions observed by a physician
Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5 % of normal control subjects
Radiographic changes	Typical changes of RA on posteroanterior hand and wrist radiographs that must include erosions or unequivocal bony decalcification localised in or most marked adjacent to the involved joints.

[1)] must be present for at least 6 weeks

Table 1: *Revised ACR criteria for classification of RA*

(cyclic citrullinated peptide antibodies) are of diagnostic and possibly prognostic value. RF is a well established marker with only limited specificity, whereas anti-CCP was introduced only some years ago. Anti-CCP is characterized by a very high specificity and a sensitivity that is at least comparable to that of RF and may be an aid in early diagnosis of RA. Disease activity of RA is usually scored by patient self-report assessment or by physician assessment.

Extra-articular manifestations

In addition to articular signs, systemic symptoms, such as fatigue, malaise, weight loss, anaemia, thrombocytosis and non-specific, diffuse musculoskeletal pain may be present. "Flu-like" symptoms are common. Main extra-articular manifestations of RA can be found in Table 2. ■

Organ	Manifestation
Blood vessels	Systemic vasculitis (small vessels)
Lungs	Pulmonary nodules
Interstitial fibrosis	
Heart	Pericarditis
Eyes	Keratoconjunctivitis sicca, Episderitis and Sceleritis
Skin	Subcutaneous nodules (rheumatoid nodules)
Immune system	Rheumatoid factor, anti-CCP antibodies
Felty's syndrome: splenomegaly, leucopenia, bacterial infection, skin ulcers	

Table 2: *Extra-articular manifestations of RA*

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Serological Diagnosis of Rheumatoid Arthritis

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The importance of early diagnosis of rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory systemic disorder with an autoimmune pathogenesis. It occurs primarily in the joints but extra-articular manifestations (e.g. vasculitis) are also possible. RA has considerable individual and social importance on account of its frequency (prevalence rate 0.5 – 1% of the population), its chronic nature (in 80-90% of cases) and its progression with the development of a variety of consequent problems. Two thirds of those affected by RA develop varying degrees of progressive structural and functional joint damage with limitation of quality of life, premature inability to work and increased mortality. However, appropriate treatment at a sufficiently early stage can have a favourable effect on progression and serious consequences. Various treatment strategies with different drug combinations have proved effective in reducing disease activity, radiological progression and limitation of everyday activities. With appropriate drug combinations it is possible to achieve remission of rheumatoid arthritis and sustained inhibition of the progression of structural and functional damage in up to 70% of patients. Such treatment can also reduce the consequences of the illness as indicated by illness-related co-morbidity and mortality. With markedly improved treatments now available, it is essential to start effective treatment as early as possible. This, of course, requires that a reliable diagnosis can also be made during the early stages of the disease's development. The clinical features of early or very early RA are frequently uncharacteristic and the classification criteria of the American College of Rheumatology (ACR) are not usually fulfilled. It is therefore necessary to find serological markers which allow reliable prediction of the development of RA as early as possible. RA-specific autoantibodies, which can be detected even in the pre-symptomatic stage, should be suitable candidates for this role.

Autoantibodies in RA – an overview

Like the other systemic autoimmune diseases, RA is characterised by the occurrence of a large number of autoantibodies against non-organ specific, but also tissue-specific antigens (Table 1). However, many of these autoantibodies have no diagnostic significance because of their low level of specificity for RA. Moreover, evaluation studies regarding the diagnostic value of individual autoantibodies may vary considerably. Those differences may result from the differing compositions of RA and control groups, differences in the source and preparation or manufacture of target antigens and the use of differing systems for the detection of autoantibodies. For example the sensivity of calpastatin autoantibodies is reported in one study as being 82% for RA while another study found a positive result in only 9% of RA patients. Likewise the diagnostic specificity reported ranges from very low to 96%. These results are, of course, not encouraging for practical purposes. However, with further investigation of these autoantibodies, results may nevertheless be obtained which have real diagnostic or prognostic usefulness.

The autoantibodies in RA can be allocated to the following groups according to their clinical relevance:

(1) The result of published studies give no indication that the autoantibody could be diagnostically useful (e.g. ANCA, autoantibodies against calreticulin and heat shock proteins).

(2) Definite evaluation is not yet possible as different studies have obtained differing results (e.g. autoantibodies against calpastatin and GPI).

(3) Findings of potential diagnostic relevance need to be confirmed (e.g. ferritin antibodies).

(4) Autoantibodies are not specific for RA but suggest an associated disorder (autoantibodies against dsDNA, Ro/SS-A, phospholipids).

(5) Autoantibodies are not specific for RA but are used as a diagnostic parameter for RA (rheumatoid factors, RA33 antibodies).

(6) Autoantibodies are highly specific for RA (autoantibodies against citrullinated proteins and peptides).

This paper aims to give a detailed picture of the relevance of the autoantibodies in the last two groups.

Autoantibodies against	Sensitivity for RA	Specificity for RA	Clinical association
Calpastatin (ACAST)	9-82%	Very low to up to 96% (also in SLE, myositis, scleroderma, Sjögren's syndrome, psoriasis and even in healthy individuals)	Involved in the destruction of cartilage because of increased calpain activity? (autoantibody blocks the inhibitory effect of calpastatin on calpains) ACAST-C27: Early diagnosis of seronegative RA if no marker antibodies for connective tissue diseases are present
High mobility group proteins (HMG-1 and -2)	48%	None	Associated with atypical ANCA and with disease activity
Citrullinated proteins or peptides (s. table 2)	70-80%	>95-99%	Specific marker for RA, also in RF-negative patients, detectable before symptoms occur
Enzymes of neutrophil granulocytes (ANCA)	18% (pANCA and atypical ANCA)	Low	None (the association with rheumatic vasculitis could not be confirmed)
Eukaryotic elongation factor 1A (EF1A)	24% (ANA-positive RA)	Low	ANA-positive Felty's syndrome
Fc fragment of IgG molecules (rheumatoid factor)	70-80%	<90% (Also in many other autoimmune diseases, infections and healthy individuals)	One of the ACR classification criteria
Ferritin	17%	97,7%	Also in seronegative RA, associated with disease severity, more frequent in male patients
Glucose-6-phosphatase isomerase (GPI)	Up to 64%	> 95% (Not reproducible)	Induce synovitis in animal model, associated with extra-articular symptoms
Histones	11%	None	Associated with vasculitis
Heat shock proteins (Hsp60)	23%	Low	None (the association with cardiovascular diseases could not be confirmed)
Cartilage proteins (collagens I-III, fibronectin)	20-70%	Low when not citrullinated, high when citrullinated	Collagen-II autoantibodies induce arthritis in mouse model
Phospholipids	20-30%	None	Evidence for thromboembolic manifestations (secondary APS)?
RA33 (A2 protein of hnRNP complexes)	26-36%	Around 90% (also in SLE and MCTD)	More specific for RA than RF, also in RF-negative patients, detectable before symptoms occur
Ro/SS-A	5-8%	None	Association with keratoconjunctivitis sicca (secondary Sjögren's syndrome?) and other extra-articular manifestations

Table 1: *Autoantibodies in rheumatoid arthritis*

Rheumatoid factors (RF)

Rheumatoid factors can be detected in the serum of 70 – 80% of patients with established RA. However the diagnostic value of RF is limited by their relatively low disease specificity. RF are often present in type II cryoglobulinaemia (up to 100%), other inflammatory rheumatic disorders e.g. primary Sjögren's syndrome (55%), SLE (15-35%), scleroderma (20-30%), MCTD (50-60%) and polymyositis (26%), and also in infections (up to 90%), chronic liver diseases (up to 70%) and tumours (up to 25%). In healthy people RF expression is dependent on age. While people aged under 60 years only rarely express RF (1-4%), the frequency of detection increases to 5-12% above 60 years and to 25% at over 70 years of age.

In spite of their limited specificity, RF continue to be the only serological criterion used by the ACR for the classification of RA. In this context it is important to note that people with diseases of differential diagnostic relevance (psoriatic arthritis, reactive arthritis, ankylosing spondylitis, gout, polymyalgia rheumatica, arthroses) do not have a higher frequency of RF than the normal population. There is also a strong correlation between RF titre and specificity for RA. In a study carried out at TU Dresden's University Hospital, high-titred RF results (> 200 U/ml) yielded a diagnostic specificity of 95% while the specificity when all RF results were included (low titres as well) was only 68%. However, higher RF titres were only found in 45% of RA patients. This respresents a major problem for differential diagnosis as the early stages of RF-negative RA are often difficult to distinguish from seronegative spondylarthropathies (reactive arthritis, Reiter's disease, anky-

losing spondylitis) or from psoriatic arthritis.
In RA patients the RF titre is largely independent of disease activity and bears only a limited relationship to the course of treatment. High-titred RF is nevertheless associated with extra-articular manifestations and rapid progression of joint destruction. In RF-positive patients, rheumatoid nodules are more common and secondary Sjögren's syndrome is more often present.
By contrast with the majority of diagnostically relevant autoantibodies, RF are mainly of the IgM isotype. Although IgG and IgA type RF can be detected in the sera of RA patients it is not clear to what extent the isotype classification of RF is helpful for diagnostic purposes. More recent investigations suggest that the determination of IgA-RF may turn out to have some diagnostic and/or prognostic value. When IgM-RF, IgG-RF and IgA-RF are present simultaneously, the specificity for RA is higher than when only one RF isotype is present alone.
RF can be detectable years before manifestation of the disease. In healthy people with high RF titres there is a 5-40 times higher risk of occurrence of RA than in RF negative people.

RA33 antibodies (hnRNP-A2 antibodies)

Autoantibodies against hnRNP complexes were first found in 1984 in patients with SLE, RA and MCTD. In 1989 autoantibodies were described which recognised a 33 kD protein from nuclear extracts of HeLa cells. As these autoantibodies were initially found exclusively in sera from RA patients the antigen was given the name RA33. In 1992 this antigen was identified as protein A2 of heterogeneous nuclear ribonucleoprotein complexes (hnRNP-A2).
RA33 antibodies are found at differing frequencies in different populations: frequencies are low in Finland and Greece and around 26-35% in western and central Europe. The occurrence of RA33 antibodies is independent of the occurrence of RF. The frequency in RF-negative RA is about 45%. RA33 antibodies can be detected at a very early stage and can therefore be a valuable aid in early diagnosis of RA. According to results available up to now there is no association with the duration, activity or stage of the disease or with RF and CCP antibodies.
The diagnostic specificity of RA33 antibodies is between 69 and 96% depending on the study. RA33 antibodies are also present in about 20% of SLE patients and 40% of MCTD patients and can occasionally be found in other connective tissue diseases. These autoantibodies are nevertheless considered to be helpful for the serological diagnosis of RA because, when marker antibodies for SLE and MCTD (dsDNA, Sm, U1-RNP) are also included, these disease entities can be distinguished serologically. As has already been mentioned for RF, the specificity here is again clearly dependent on the autoantibody titre. In the Dresden sample a specificity of 88% with a sensitivity of 26% was found when low-titred results were included. If the moderate- and high-titred results are evaluated alone the specificity increases to 98% and the sensitivity is reduced to 12%. For results > 200 U/ml the specificity reaches 100%, but only 2.5% of RA patients are then included. It was also found that the simultaneous presence of RF and RA33 antibodies is highly specific for RA.

Autoantibodies against citrullinated proteins or peptides

History
RA specific autoantibodies against antigens in keratohyaline granules of buccal mucosa cells, so-called **antiperinuclear factors (APF)**, had already been described in 1964. In 1979 antibodies against antigens of the corneal layer of the rat oesophagus were discovered and were called **anti-keratin antibodies (AKA)**. However these antibodies do not recognise cytokeratins but rather epitopes of certain variants of epidermal filaggrin. In spite of conspicuous similarities, AKA and APF were considered for a long time to have different autoantibody specificities. Only when the APF antigen had been characterised was it possible to show that APF and AKA are similar autoantibodies, though not absolutely identical in their epitope recognition. They recognise epitopes of epidermal filaggrin and/or epitopes of (pro)filaggrin-like proteins from buccal epithelial cells (**anti-filaggrin antibodies, AFA**). A decisive step for the development of new, RA-specific assays was the discovery that the target structures recognised by APF, AKA and AFA are generated by a specific post-translational modification (conversion of the amino acid arginine into citrulline as a result of the activity of peptidylarginine deiminase, see Fig. 1).

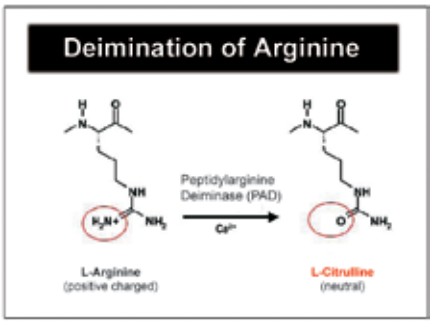

***Fig. 1:** In the generation of epitopes relevant to RA, arginine is converted into citrulline by peptidylarginine deiminase*

By targeted selection of synthetically produced citrullinated peptides (cyclic citrullinated peptide - CCP) it has been possible to establish highly sensitive and specific enzyme immunoassays for RA diagnosis. Subsequently, further citrullinated proteins (such as fibrin/ fibrinogen, type I and II collagen, vimentin) were identified as target structures of RA-specific autoantibodies.

Antiperinuclear factors (APF)
APF are autoantibodies against antigens in the keratohyaline granules of buccal mucosa cells. As early as 1964 they were described by Nienhuis and Mandema as being highly specific for RA. In further studies diagnostic specificities of between 88 and 99% and sensitivities of between 36 and 59% were found. However, in spite of its higher RA specificity by comparison with rheumatoid factors, this test failed to find a place in routine practical use because of the lack of reproducibility.

Anti-keratin antibodies (AKA)
AKA target the filaggrins/ profilaggrins of the corneal layer of rat oesophagus. The specificity for RA is between 73 and 99% depending on the study. However, most studies report a high diagnostic specificity (>95%). In the Dresden sample the specificity was found to be 96% with a sensitivity of 51%. If the low-titred results (< 1:40) are excluded, the specificity increases to > 99% and the sensitivity is simultaneously reduced to 28%. With adequate testing and investigatory procedures, AKA are thus highly specific for RA. The sensitivity, which is between 33 and 54%, is however markedly below that achieved by enzyme immunoassays for antibodies to CCP, MCV and citrullinated filaggrin. AKA can be detected in about 30% of RF negative RA patients.

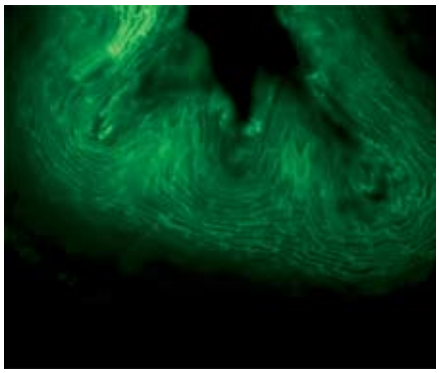

Fig. 2: Typical laminar fluorescence of stratum corneum of rat oesophagus (unfixed cryostat sections of the middle third) with presence of antikeratin antibodies (AKA).

AKA determination was, and continues to be, performed in only a few laboratories. Even before the CCP era, this test had scarcely found

Autoantibodies against citrullinated proteins/peptides	Sensitivity for RA	Specificity for RA
APF (antiperinuclear factor)	36-59%	88-99%
CCP (cyclic citrullinated peptide)	68-85%	91->99%
EBNA-1 (VCP: viral citrullinated peptide)	45-50%	>95%
Fibrin(ogen) (α- and β-chain)	56%	93%
Filaggrin	47-51%	90-99%
Collagen type I (α1- and α2-chain)	19-32%	99%
Collagen type II (α1-chain)	40%	>95%
MCV (mutated citrullinated vimentin)	75-82%	92-97%
Vimentin (Sa)	20-47%	98-100%
AKA (antikeratin antibody)	36-59%	73-99%
α-Enolase	46%	No data

Table 2: *Autoantibodies against citrullinated proteins or peptides*

its way into routine diagnostic use in spite of its high specificity for RA. AKA are only rarely found in RA patients negative for CCP antibodies (less than 5% overall in the Dresden study and only 2% with a titre of > 1:20). This means that AKA determination is only indicated in patients with possible or probable RA who are negative for CCP antibodies.

Anti-Sa antibodies

Patients with RA were shown in immunoblot analysis to possess serological reactivity to an approx. 50 kD protein from human spleen, placenta and synovia. This protein is present in abundance in the placenta and synovia of RA patients. Because the antigen was at first unknown, the new antibody was named after the index patient (Sa). It could be shown that these anti-Sa antibodies can be used as diagnostic markers for RA with a sensitivity of between 22 and 40% and a very high specificity of 98 to >99%. Anti-Sa antibodies indicate a prognosis of arthritis taking a severe course. They are also present in RF-negative RA patients. In spite of these impressive results, the determination of anti-Sa antibodies has not established itself in RA diagnosis, mainly because of the lack of standardisation of the assay. It has recently been shown that the main target antigens of anti-Sa antibodies are citrullinated forms of vimentin (see also MCV antibodies). In the Dresden sample we were able to show that anti-Sa antibodies are also present in about 9% of RA patients negative for CCP and MCV antibodies.

Antibodies against citrullinated filaggrin (anti-filaggrin antibodies, AFA)

AFA have been found in 47-51% of RA patients. The specificity for RA was reported as being between 90 and 99%. The Dresden study found a very high RA specificity, although with lower sensitivity (64%) by comparison with CCP and MCV antibodies. Of RA patients negative for CCP antibodies, 8% were positive for AFA.

Anti-CCP antibodies

CCP stands for cyclic citrullinated peptide. Since it became known that the specificity of APF, AKA and filaggrin antibodies for RA depends on the generation of citrulline-containing epitopes (see Fig. 1), numerous synthetically produced citrullinated peptides have been tested for their applicability in rheumatoid serology. This led to the development of the CCP antibody assays (currently in the second generation). It was quickly shown that CCP antibodies are far superior to RF in terms of specificity. Depending on the study, specificities for RA were between 91 and over 99% and sensitivities were between 68 and 85%. In the Dresden study we found a specificity of 96% and a sensitivity of 76%. When only moderate- and high-titred results were evaluated the specificity rose to 98% and the sensitivity was reduced to 72%. Anti-CCP antibodies can also be found in about half of RF-negative RA patients. They already occur frequently (more frequently than RF) in early stages of the disease or even pre-symptomatically (up to 10 years before clinical manifestation). Furthermore they act as prognostic markers for the development of erosive RA. An association between the antibody titre and clinical activity is being discussed. CCP antibodies are only rarely found in other arthropathies, connective tissue diseases or other disorders. The development of RA should be borne in mind whenever they are present at high titres and the relevant monitoring should be undertaken.

Anti-MCV antibodies

MCV stands for mutated citrullinated vimentin. The Dresden study found a specificity for RA of 92% with a sensitivity of 75%. When low-titred results were excluded the specificity was 98.5% and sensitivity was reduced to 61%. Of RA patients negative for CCP antibodies, 21.4% were positive on the MCV test although mostly with lower titres.

Autoantibodies as predictive markers for the development of RA

As RF and autoantibodies against RA33 and citrullinated proteins or peptides can be detected years before clinical manifestation of RA, these antibodies could also be helpful for predicting development of the disease and could thus serve as the earliest possible diagnostic tool. A precondition for such an application would be that predictive values were high. However the positive predictive values (PPV) for RF and RA33 antibodies are well below 90%. High PPV of >95-100% are, however, achieved when high-titred RA33 antibodies are present and also with moderate- to high-titred results for all autoantibodies against citrullinated proteins/ peptides and with combinations of at least 2 of the autoantibodies described, with the exception of the combination of RF and RA33 antibodies. The negative predictive values (NPV) for these constellations are between 42 and 77%.

Recommendations for diagnostic procedures

Even though autoantibodies against citrullinated proteins or peptides have not up to now been included in the classification criteria for RA, and are, in part, not yet accepted by health insurance funds as important diagnostic markers, they should always be determined for screening purposes where RA is suspected. Determination of CCP antibodies is to be recommended, even in cases already found to be positive for RF, because the combination of both autoantibodies is absolutely specific for RA. This makes it easier to reach decisions on treatment especially in early forms of RA. The determination of both RF and CCP antibodies is recommended by the German Rheumatology Association (Deutsche Gesellschaft für Rheumatologie, www.dgrh.de) and the working group on autoimmune diagnostics of the German Association for Clinical Chemistry and Laboratory Medicine (Deutsche Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, www.dgkl.de).

Because RA33 antibodies and other autoantibodies against citrullinated proteins (AKA, antibodies for Sa, filaggrin and MCV) are more specific than RF for RA, and since efforts are being made to achieve appropriate early treatment, these parameters should be determined

in individuals negative for CCP antibodies. It would be helpful to produce autoantibody profiles including all the parameters described to increase the sensitivity and specificity of RA diagnostics. This would make it possible in future for more patients to be given appropriate treatment at an early stage. In the Dresden study we were able to show that, when all parameters are combined, the sensitivity at a titre yielding a specificity of >98% (e.g. AKA > 1:20, RA33 antibodies from moderate titres) can be improved to 88%. Certain antibody combinations were also evaluated as being absolutely specific for RA, such as the combination of CCP antibodies with AFA or RF or RA33 antibodies, the combination of moderate-titred RF with RA33 antibodies or the combination of MCV and RA33 antibodies.

Outlook

Genome and proteome research will certainly throw up new candidates for serological RA diagnostic procedures. At present attention is being focussed on the identification of further citrullinated target antigens (Table 2). The development of new multiparametric assays will make it possible to determine 10 or more autoantibodies in a single test run. With appropriate combinations of autoantibodies, not only the sensitivity and specificity but also the predictive values for RA could be increased. Such combinations would then lend themselves for use in predictive diagnosis. Furthermore it would be desirable to be able to make use of autoantibodies or autoantibody combinations as prognostic markers when making decisions on different forms of treatment. ■

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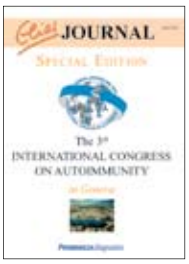

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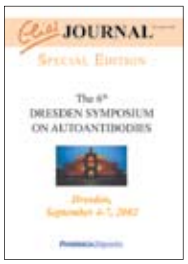

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