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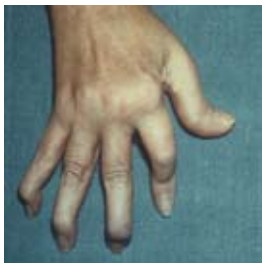

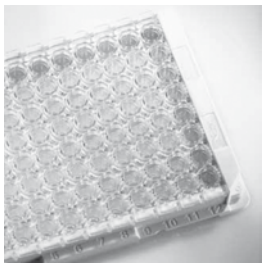

Phadia

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Editorial

The term lupus (Latin for wolf) is attributed to the thirteenth century physician Rogerius who used it to describe erosive facial lesions that were reminiscent of a wolf's bite. The Frenchman Cazenave, in 1851, was the first one to apply the term Lupus erythematosus. In the following 100 years, the disease was described several times from different points of view. Jadassohn in Vienna and Osler in Baltimore had established systemic lupus erythematosus (SLE) as a separate disease entity by the turn of the century. However, considerable confusion still remained because many thought SLE was a variant of tuberculosis and even typical cases of SLE were reported under a variety of names. Today, SLE is still often misdiagnosed because of the high variety of its clinical presentations. On page 3 Professor Erika Gromnica-Ihle from the Rheumaklinik Berlin-Buch, Berlin, reviews the clinical picture and treatment of SLE in her article.

In 1948 Hargraves, Richmond, and Morton described the "LE cell" in the marrow of SLE patients. The test was later adapted to peripheral blood. This single discovery revolutionized our ideas of SLE. In 1957 an American physician George Friou, applied the indirect fluorescent technique to the study of autoantibodies (FANA). At about the same time, the first SLE-specific antibodies, anti-dsDNA and anti-Sm were described. Later, this made the development of specific enzyme linked immunosorbent assays (ELISA) for the detection of antinuclear antibodies possible. On page 10 Graham Wood describes the experiences of the Clinical Immunology Laboratory at Addenbrookes Hospital, Cambridge, UK when they changed their laboratory routine screening from FANA to Varelisa. Professor Hans C. Nossent from the University Hospital of Tromsø, Norway, provocatively asks on page 11 if there is still a future for FANA or if a change to specific autoantibody testing is inevitable.

The specificity of ELISA ANA testing is improving continuously. One of the first ANAs described, the Sm antibody, is useful for the diagnosis of SLE because of its high specificity for the disease. The Sm antigen is composed of at least nine different polypeptides. However, the high specificity for SLE is only achieved when the so-called SmD polypeptides are used. Unfortunately, purified SmD is always at risk of being contaminated with other Sm polypeptides, and thus may be not specific enough. Until now, all trials to produce a recombinant SmD protein with a good reactivity have failed. In 2004, Mahler et al. developed an ELISA based on a synthetic SmD peptide which showed an exceptionally high sensitivity and specificity for SLE. In his article on page 7, Dr. Michael Mahler introduces this synthetic SmD peptide. Clearly convinced of the innovative character and diagnostic usefulness of this antigen, Pharmacia Diagnostics reworked the Varelisa Sm Antibodies assay and the Varelisa ANA Profile assays, which now use the SmD peptide in order to achieve a significant improvement of clinical efficiency in the diagnosis of SLE.

Pleasant reading

Nina Olschowka

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The clinical picture and treatment of systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a disease of unclear aetiology, in which tissue and cells are damaged by pathogenic autoantibodies and immune complexes. 90% of patients are women, mostly of child-bearing age but children, men and older people can also be affected. The incidence of SLE has tripled in the last 40 years and now totals 7.3 per 100 000 population per annum (1). Prevalence data in research varies between 15 and 50 per 100 000 population (2) depending on case-finding methods and ethnic differences. Non-Caucasians are disproportionately affected. In central Europe prevalence is estimated at 12.5 in 100 000 women. Early diagnosis, better SLE therapy and optimisation of the treatment of comorbidity have led to marked improvement in patients' chances of survival. Today over 90% of patients survive for 10 years (3).

Clinical Signs of SLE

The degree of severity of SLE varies greatly. Milder forms of the disease with recurrent symptomatology, persistent or fulminating forms are being observed. Most patients experience exacerbations interrupted by relatively asymptomatic periods. Total remission without any symptomatology occurs only very rarely without therapy.

Common general symptoms of SLE are poor performance, exhaustion, general malaise, fever, anorexia and weight loss.

The frequency of the clinical symptoms of this multiorgan disease is shown in figure 1. The EURO-Lupus project (4), a prospective study, has provided exact numerical data over a ten year period (table 1).

Musculoskeletal System: Almost all patients show signs of arthralgia and myalgia; most develop intermittent arthritides. These are characterised by the absence of joint destructions. However, joints can become deformed as in Jaccoud's arthritis (figure 2). Tenosynovitides, rheumatoid nodules and spontaneous osteonecrosis are also observed.

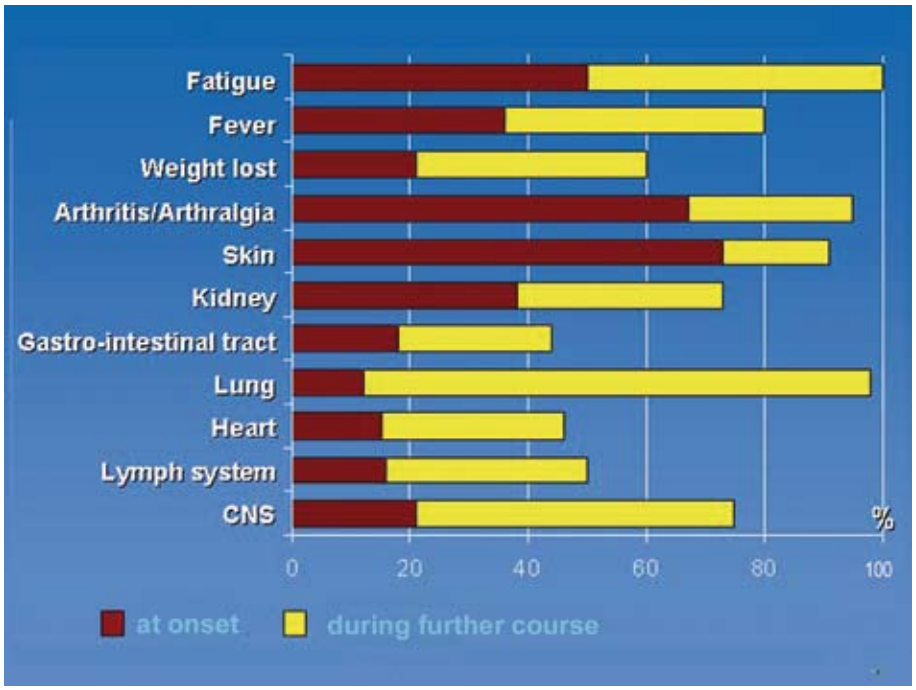

Figure 1: *Frequency of clinical symptoms of systemic lupus erythematosus*

Symptom	Frequency in percent
Arthritis	48
Butterfly erythema	31
Active nephropathy	28
Fever	17
Neurological symptoms	19
Raynaud's phenomenon	16
Serositis	16
Thrombocytopenia	13
Thromboses	9
Myositis	4

Table 1: *Prospective study with 1000 patients over a period of 10 years (Euro-Lupus-Project)*

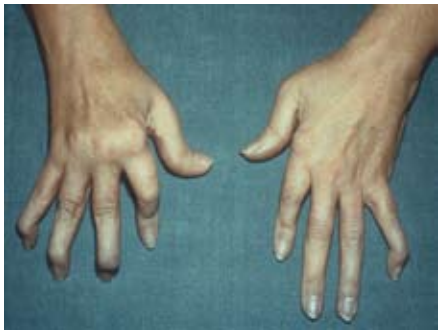

Figure 2: *Jaccoud's arthritis in a case of systemic lupus erythematosus*

Skin: Sontheimer has classified the various different skin manifestations of lupus erythematosus.

Chronic cutaneous lupus, which can be localized or disseminated, is mostly observed on scalp, ears, face, and on those areas of the arm, back, and collar that are exposed to the sun. Only 5% of these patients develop SLE. Chronic cutaneous lupus causes scarring.

Subacute cutaneous lupus erythematosus, which exhibits a papulosquamous or annular polycyclic morphea without scarring, frequently accompanies arthritis and poor performance. CNS and kidney manifestations are extremely rare. Some of these patients show no signs of antinuclear antibodies (ANA). Most possess antibodies against Ro (SS-A).

Acute cutaneous lupus manifests itself as butterfly erythema (figure 3) on the face or as a more diffuse macular papular erythema on other areas which are exposed to the sun. In most cases it indicates an episode of SLE. Non-specific skin changes like vasculitic lesions, alopecia and urticaria can also occur. Nasopharyngeal or oral mucosal ulceration is another common symptom.

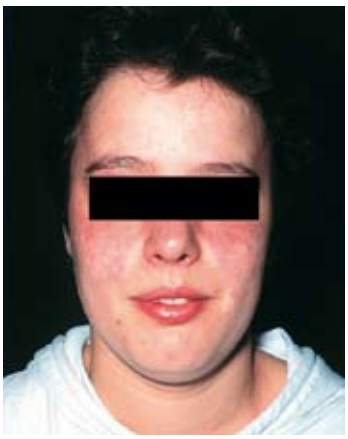

Figure 3: *Butterfly erythema*

Kidneys: Most SLE patients display immunoglobulin deposits in the glomeruli, but only half of SLE patients show clinical signs of nephritis characterized by proteinuria. During the early stages most patients are clinically asymptomatic. Urine analysis indicates erythrocyturia, cylindruria and proteinuria. The classification of lupus nephritis is presented in table 2. A renal biopsy gives useful information relevant to the treatment of SLE and should always be conducted, if therapeutic decisions are to be made on the basis of the occurrence of nephritic urine sediment. Most patients with mesangial or mild focal proliferative nephritis retain good kidney function. Without treatment, diffuse proliferative nephritis leads to kidney failure.

Heart/lungs: Cardiac manifestations of SLE are: pericarditis (30%), endocarditis (10%), myocarditis (10%) and coronary arteritis (8%). Pleurisy and pleural effusions are very common manifestations of SLE. Lupus pneumonitis causes fever, dyspnea and coughing. X-ray examination of the thorax reveals migratory infiltrates and/or areas with plate atelectasis. Pulmonary hypertonia occurs in fewer than 5% of patients and can have a significant impact on a patient's life expectancy.

Nervous system: There is broad spectrum of potential neurological complications. Psychoses, epilepsy, organic brain syndrome, stroke, paralysis of cranial nerves, non-bacterial meningitis, migraines and peripheral neuropathy are some of the main clinical symptoms. Mild cognitive dysfunction is also common.

I Normal result
II Mesangioproliferative glomerulonephritis
III Focal proliferative glomerulonephritis
IV Diffuse proliferative glomerulonephritis
V Membranous glomerulonephritis
VI Sclerosing glomerulonephritis

Table 2: *Classification of nephritis associated with lupus*

Clinical criteria

1. Vascular thrombosis
   - ≥ 1 clinical episodes of arterial, venous or small vessel thrombosis (confirmed by imaging or Doppler studies or histopathology)
2. Pregnancy morbidity
   - ≥ 1 unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, or
   - ≥ 1 premature births of a morphologically normal neonate at or before the 34th week of gestation, or
   - ≥ 3 unexplained consecutive spontaneous abortions before the 10th week of gestation

Laboratory criteria

1. Anticardiolipin antibody of IgG and/or IgM isotype in blood, present in medium or high titre, on 2 or more occasions, at least 6 weeks apart, measured by a standardised ELISA for ß2GP1-dependent anticardiolipin antibodies.
2. Lupus anticoagulant present in plasma, on 2 or more occasions at least 6 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Hemostasis, in the following steps:
   - a) Prolonged phospholipid-dependent coagulation demonstrated on a screening test, e.g. PTT, KCT, dRVVT, Textarin-Test
   - b) Failure to correct the prolonged coagulation time on the screening test by mixing with normal platelet-poor plasma.
   - c) Shortening or correction of prolonged coagulation time on the screening test by the addition of excess phospholipid.
   - d) Exclusion of other coagulopathies, e.g. factor VIII inhibitor or heparin, as appropriate.

The diagnosis of APS is based on at least one clinical and one laboratory criterion being fulfilled.

Table 3: *Sapporo criteria for the classification of antiphospholipid syndrome*

Haematopoietic system: Most patients with active SLE develop anaemia. In a very small percentage of patients the anaemia is Coombs' test positive. Leukopenia (mostly lymphocytopenia) is also common. However, it is only rarely attended by recurrent infections and does not usually require treatment. Mild thrombocytopenia is common; severe thrombocytopenia with bleeding is fairly rare but can nevertheless be an initial symptom of SLE.

Gastrointestinal tract: Common gastrointestinal symptoms are nausea, diarrhoea and a non-specific malaise. The symptoms may be due to lupus peritonitis and signal a lupus episode. Vasculitis of the intestine is a very dangerous complication. It is accompanied by acute convulsive abdominal pains, vomiting and diarrhoea and can even lead to bowel perforation. Some patients show motility disorders similar to those of systemic sclerosis.

Vessels: Thromboses in veins, arteries, even in microscopic vessels can be a serious problem. Vasculitis may be the cause of the thrombosis but, in most cases, can be put down to antiphospholipid syndrome. This is defined by the criteria shown in table 3. Additionally, degenerative changes occur in vessels after an extended period (years) of exposure of blood vessels to circulatory immune complexes. Further, the hyperlipemia brought about by corticosteroid treatment leaves lupus patients predisposed to cerebral- and coronary arteriosclerosis.

Table 4 shows the classification criteria for SLE. In fully developed cases of the disease these criteria allow doctors to make a good diagnosis. The real difficulties lie in the early diagnosis of SLE.

Various different subsets indicate particular forms of SLE and how they overlap with other diseases (figure 4).

Treatment

There is, as yet, no cure for SLE. The aims of SLE therapy are therefore to control symptoms, to prevent the disease from progressing and to reduce pathological changes in the organs. A benefit-risk analysis should always be conducted to avoid causing any damage during treatment. The treatment of SLE depends on the nature and the severity of organ manifestations. 25% of SLE patients suffer from a mild form of the disease and do not need any immunosuppressive therapy. Table 5 presents an overview of medicinal treatments as indicated by organ manifestations.

80% of SLE patients are treated with non-steroidal antirheumatic drugs (NSAR). Arthralgias, arthritis, myalgias, fever and low-grade serositis all respond well to NSAR. However, gastric ulcers, kidney damage, NSAR-induced hepatitis, aseptic meningitis, allergic reactions and an increase in photosensitivity may be observed (the last, particularly in the case of SS-A antibody positive patients). The role of specific

1.	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2.	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3.	Photo-sensitivity	Skin rash as a result of unusual reaction to sunlight, by patient's history or physician's observation
4.	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5.	Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion
6.	Serositis	Pleuritis – convincing history or pleuritic pain or rub heard by a physician or evidence of pleural effusion, or Pericarditis – documented by ECG or rub or evidence of pericardial effusion
7.	Renal disorder	Persistent proteinuria > 0.5 g/d or >3+ if quantitation not performed, or Cellular casts – may be red cell, hemoglobin, granular, tubular or mixed
8.	Neurologic disorder	Seizures or Psychosis – in the absence of offending drugs or known metabolic derangements; eg uremia, ketoacidosis, or electrolyte imbalance
9.	Hematologic disorder	Hemolytic anemia – with reticulocytosis, or Leukopenia (<4000/mm3), or Lymphopenia (<1500/mm3, or Thrombocytopenia (<100000/mm3) in the absence of offending drugs
10.	Immunological disorder	Anti-dsDNA-, anti-Sm- or anti-phospholipid-antibody
11.	Antinuclear antibodies	An abnormal ANA titre by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug induced lupus" syndrome.

Table 4: *Criteria for the Classification of systemic lupus erythematosus first published in 1982; updated in 1997 (6, 7). For the classification of SLE, 4 or more of the 11 criteria have to be present, serially or simultaneously, during any interval of observation (specificity: 98%, sensitivity: 97%).*

COX-2-inhibitors has so far not been researched for treating SLE.

Since the Canadian hydroxychloroquine study in 1971, the antimalarial drug has been known to reduce the disease activity and frequency of episodes of the arthralgia and skin manifestations of SLE (8). The superiority of chloroquine over the placebo was confirmed by further studies (9). Additional advantages of treatment with today's preferred (and related) drug, hydroxychloroquine, are reduced cholesterol, glucose and triglyceride (10,11), an antithrombotic effect in cases of secondary APS (12) and reduced photosensitivity. Side effects such as retinopathy, exanthema, myopathy and neuropathy are rare. Regular ophthalmologic checks should be conducted at least once a year.

The most significant part of SLE therapy is the use of corticosteroids (CS). Table 6 provides an overview of initial dosage as dependent upon the disease severity and organ involvement. With a higher disease activity, the daily CS dose should be divided so that it is administered every 8 - 12 h. Patients experiencing an acute episode, and patients suffering from severe lupus nephritis in particular, can be treated with a course of methylprednisolone "pulse therapy" (1000 mg/d), administered intravenously for 3 – 5 days. Once the disease is under control, it is recommended that the CS dose be taken once daily in the mornings and that the daily dose is thereafter reduced as quickly as the patient's condition allows. The undesired effects of CS treatment must always be taken into consideration. Primarily, these include osteoporosis, avascular osteonecrosis, progressive arteriosclerosis and reduction in quality of life resulting from weight gain and Cushing's disease as well as the increase in susceptibility to infection. In order to minimize the osteoporosis, patients should take calcium (1 000 mg/d). Vitamin D is also useful. Those patients with more severe forms of SLE may require permanent treatment with CS .

Azathioprine is used in SLE treatment to lower the requirement for high CS dosage. It is also used in the case of an initial life-threatening organ manifestation and in sustaining remission following cyclophosphamide therapy. Azathioprine is given in doses of between 2 and 3 mg/kg/d. White blood counts should not fall below 3 x 10^9/l. Interactions with allopurinol and marcumar should be monitored.

Smaller randomised studies in recent years (13,14) confirm that a dose of MTX is effective in SLE treatment. Its effect consists primarily in influencing symptoms affecting the locomotor apparatus.

Leflunomide is ineffective in severe cases of SLE; arthralgias are positively influenced (15).

Indications for cyclophosphamide therapy are: active lupus nephritis in stage II, IV and possibly also V, as well as life-threatening organ complications that are not responsive to CS or azathioprine. Doctors should always consider a critical benefit-risk analysis. Amenorrhoea and malignancies are the most important side effects to take into consideration. Today cyclophosphamide therapy for SLE is generally carried out as pulse therapy. It is administered monthly in doses of 500 to 1000 mg/m² body surface area until remission, or for up to six months. Subsequently, the same dose is infused at 3-month intervals for a maximum of 18 months. The addition of methylprednisolone on the day of cyclophosphamide pulse therapy can improve the effectiveness of the cyclophosphamide (16).

There have been repeated attempts to try to avoid the toxic effects of cyclophosphamide. In one small study mycophenolate mofetil demonstrated the same effectiveness in treating lupus

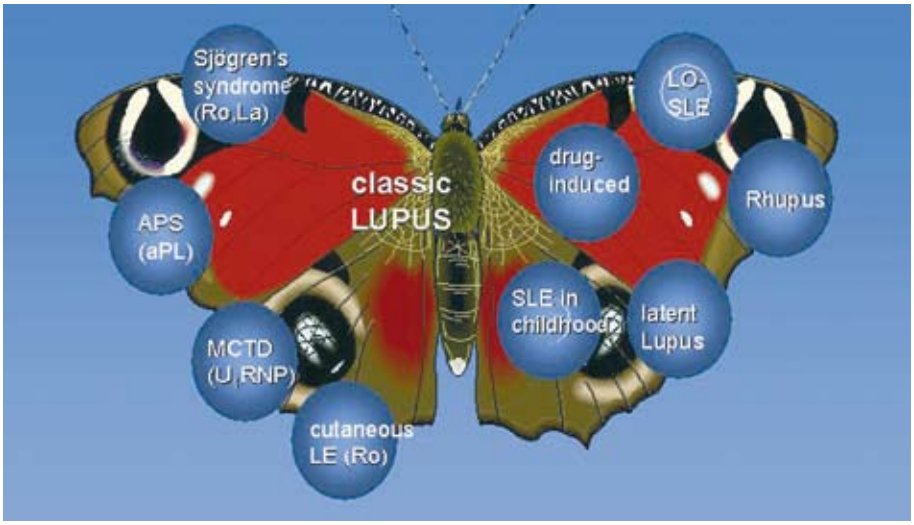

Figure 4: *Subsets of systemic lupus erythematosus; Key: LO - SLE = late-onset SLE; Rhupus = rheumatoid arthritis + SLE; MCTD = mixed connective tissue disease; APS = antiphospholipid syndrome*

Drug	SLE-manifestation				
	General situation	Joints/ muscles	Skin	Serositis	Visceral organs
NSAR	+	+		+	
Corticosteroids (topical)			+		
Corticosteroids (low potency)	+	+	+	+	
Corticosteroids (high potency)					+
Anti-malaria drugs	+	+	+	+	
Azathioprine	+	+	+	+	+
MTX		+	+		
Leflunomide		+			
Dapsone			+		
Thalidomide			+		
Cyclophosphamide					+
Cyclosporine					+
IVIG					+

Table 5: *Overview of therapies for systemic lupus erythematosus*

nephritis as cyclophosphamide, and with fewer side effects (17). However, a monitoring study of this group found that patients who had gone into remission following treatment with mycophenolate mofetil showed a higher chance of relapsing into glomerulonephritis when receiving maintenance therapy with azathioprine as those who went into remission following treatment with cyclophosphamide (18). One recent study (19) was able to prove that, of the three types of maintenance therapy used to sustain a remission achieved through monthly cyclophosphamide pulses, (namely the continuation of three-monthly cyclophosphamide therapy, mycophenolate mofetil or azathioprine therapy) these last two were more effective and had fewer side effects.

Cyclosporin A can also bring about a significant improvement to SLE activity. It is effective in the treatment of pancytopenia and thrombocytopenia (20) and membrane nephritis (21). Cyclosporin A can also be tried where there is resistance to cyclophosphamide.

Recently, reasonable success has been achieved treating severe forms of SLE using stem cell transplantation (22). A high dosage course of cyclophosphamide without stem cell transplantation seems, potentially, to be equally effective (23).

A great number of monoclonal antibodies are currently undergoing clinical trials in the treatment of SLE. The value of immunoabsorption is likewise yet to be proved.

Further therapeutic measures not specific to SLE are also significant, such as the abovementioned mandatory osteoporosis prophylaxis or therapy during the administration of CS. Hypertonia therapy must be well designed. A reduction in lipids is obligatory if values are raised.

Finally, SLE patients should always be given detailed advice about avoiding direct sunlight and using sun creams, about planning of possible operations and pregnancies, immunization and contraception. Advising patients in groups, preferably in their self-help group, has proven successful.

In conclusion, the presentations and complications of SLE are many and varied. Clinicians should always be aware of the possible exacerbations of the disease and consider carefully the potential side effects of prescribed therapy. SLE patients require thorough, individualised patient management.

SLE-picture	Organ involvement	Dosage in mg/kg KG	Dosage interval/d
Mild	Skin, joints, muscles	0.25 – 0.50	once
Medium to severe	Skin, joints, haematological manifestation, serositis, mild renal manifestation	0.5 – 1.0	split in 2-3 doses
Severe, life-threatening	Severe lupus nephritis, CNS involvement, severe serositis	1.5	every 8 hours

Table 6: *Corticosteroid therapy for systemic lupus erythematosus*

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Living with ANA ELISA assays - the Cambridge experience

Graham Wood

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The Clinical Immunology Laboratory at Addenbrookes Hospital, Cambridge is a large regional unit which serves a population of 4 million people in East Anglia. At the end of 2002 as part of a regular review of laboratory methodology and procedures we decided to review Anti Nuclear Antibody (ANA) testing at Addenbrookes. At this time we were processing approximately 25,000 samples for ANA testing by a traditional Indirect Fluorescent Assay(IFA) technique using HEp-2 cells and an automated slide processor. This ANA testing procedure required 1.0 whole time equivalents (WTE) BMS/MLA to set the tests up and perform the initial reading of the slides on the fluorescent microscope, and 0.2 WTE of a senior BMS to re-read the slides (The double reading of IFA was introduced as a result of an audit which showed that single reading of IFA gave significant numbers of reading and clerical errors, which were virtually eliminated by the second reading process)

The questions which arose from this review were:

- Were the resources (staff and time) spent on ANA testing appropriate?
- Were there alternative technologies available for ANA testing?
- Would these alternative techniques offer the same or similar clinical utility?
- Would they offer operational advantages to the laboratory?
- Would they be affordable?
- Would a change be supported by the clinical users?

We first reviewed the evidence base behind the clinical use of anti nuclear antibody testing. A literature review and a series of meetings with the clinical users of the service confirmed that ANA testing is most useful for ruling out systemic rheumatic disease rather than ruling it in. (i.e. making use of the high sensitivity of the test.)This certainly seemed to fit with the fact that the number of ANA tests we were performing was 50 fold higher than the incidence of systemic rheumatic diseases would suggest. The high sensitivity was at the expense of poor specificity with 30% of ANA positives by IFA not having any clinically significant autoantibody detected, leading to needless referral to rheumatology clinics. We therefore concluded that to offer the most efficient testing regime, we needed to use a test which had better specificity for the diseases in question rather than the traditional HEp-2 IFA approach. The most suitable assay system was an ELISA assay utilising a restricted set of nuclear and cytoplasmic autoantigens which would allow detection of the most clinically significant ANA autoantibodies, namely dsDNA, RNP,Sm,Ro,La,Scl-70 and Jo-1.

At the end of 2002 we undertook an evaluation of the Pharmacia ReCombi ELISA assay, comparing 92 samples of defined clinical status and antibody profile and 250 consecutive samples tested using the current IFA technique. The results of the study showed that the detection of the most clinically significant autoantibody specificities in systemic rheumatic disease by ELISA was similar to that using IFA. We discussed the finding of our study, with the clinical users and, with their agreement and encouragement, made the change to the ELISA format in February 2003. This was done on the basis that HEp-2 would still be available if specifically requested, that information about the change would be made available to users and that regular review and audit would take place.

So, two years later what have been the advantages to the laboratory?

1. ELISA technology does not require the same degree of operator skill as for IFA so we were able to redeploy the more skilled staff to other areas of the laboratory where more manual or complex assays were being performed. This is very important as, at Addenbrookes and most other NHS Trust laboratories, the recruitment and retention of Biomedical Scientists is problematic, with little prospect of improvement.
2. Fewer follow-up tests are performed as our ANA positive rate was halved with the change to ELISA.
3. ELISA technology is more reproducible and easier to control than IFA in our hands.
4. Less risk of result transcription error with fully automated and interfaced EIA processor.
5. Reduced sample manipulations as follow-up tests are on the same platform as the screening test.
6. Turn-around times have improved – using EIA we can easily process 300 samples per day if necessary.

And to the clinical users of the service?

1. Fewer ANA "false" positives leading to fewer clinical dilemmas and lower referral rates.
2. ELISA results provide more concise, objective and easy-to-interpret information than was being given by the IFA assay.

It is also interesting to note that in the 2 years since we changed to EIA we have had only 5 or 6 requests to perform HEp-2 out of over 55,000 requests for ANA.

What are the disadvantages?

1. Increased reagent costs: There was a modest increase in reagent costs (£6.5K) but this was more than offset by the savings in staff time (£16K).
2. The ELISA assay uses a limited panel of autoantigens and so is negative in patients with the more infrequently seen autoantibodies such as ribosomal P and PM-Scl. The clinical users however, feel that this is more than offset by the lower rate of false positives and that generally these patients will be diagnosed on clinical criteria anyway.

So, have we found the answers to the questions we asked during our review of ANA testing in 2002?

Yes we have. For our laboratory service, ELISA technology can reliably detect the most clinically significant Anti Nuclear Antibodies in a cost effective manner, allowing more efficient use of skilled laboratory staff and at the same time improving the clinical utility of the test for our users.

At regular review meetings with the clinical users of the service, satisfaction ratings are very high, and so, consequently, we have no plans to return to IFA.

Antinuclear antibody testing: Is there a future?

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Antinuclear antibodies (ANA) are intriguing constituents of the immunologic apparatus, which are known to interact with a wide range of essential intracellular macromolecules (including DNA, RNA, proteins and ribonucleoprotein complexes), that are responsible for maintaining the orderly functioning of living cells. While our knowledge about the triggers for ANA production as well as the consequences of ANA presence is limited, some of the ANA are, to a certain extent, associated with connective tissue diseases (CTD)(1;2). This has led to the common view that ANA are excellent diagnostic markers for rheumatologic disease in general and has resulted in a strikingly inappropriate use of ANA testing in the diagnostic workup of patients with musculoskeletal complaints (3).

Finding of a positive ANA test in patients without objective findings of a distinct CTD is not only a futile but also potentially dangerous exercise, as such patients may be unnecessarily referred to specialised centres and/or exposed to trial therapies based on the presence of ANA (4). On the other hand, while a negative ANA screening test makes the presence of a disorder such as systemic lupus erythematosus (SLE) unlikely, it does not exclude the presence of other potentially serious CTDs, for which different investigations are indicated. It is important to realize that the presence of unspecified ANA is not a prerequisite in CTD classification (in practice: diagnostic) schemes other than SLE. In the light of its poor specificity and poor sensitivity for diseases other than SLE, it is thus difficult to understand how broad ANA testing can still be recommended when the suspicion of any type of systemic autoimmune disease is raised (8).

Classification criteria sets for CTDs use the accumulated and documented presence of a certain number and type of clinical conditions to categorize patients into distinct disease groups. They are commonly based on statistical analyses of retrospective long-term observations and solely intended for use in research settings. Typically these criteria sets do not require the simultaneous presence of certain ANA and clinical symptoms (a prerequisite for a diagnostic test), but yet they are often – and incorrectly – applied as diagnostic criteria in clinical practice. Some classification criteria sets for CTD currently require the presence of specific autoantibodies, such as the presence of anti-SSA antibodies in the current criteria for Sjøgren's syndrome, high titers of anti-RNP antibodies in the MCTD-criteria set and the sustained presence of one of three more-or-less defined antiphospholipid antibodies (APL Ab) in the antiphosholipid syndrome (APS) (5-7). The presence of unspecified ANA has, for a long time, been one of 11 criteria used in classification of SLE patients (8). Based on the above, it is clear that if broad ANA testing is applied, it should be restricted to cases where SLE is considered. In view of the more specific ANA tests required for the other CTDs and, given the sensitivity of current ELISA techniques, one may speculate if the presence of more specific autoantibodies such as anti-dsDNA Ab and anti-Sm Ab should replace the broad use of ANA in SLE classification as well (9).

Much of the confusing puzzle of ANA testing is based on methodology. Indirect immunofluorescence to detect ANA (F-ANA) is the prevailing ANA screening test around the world and is largely performed in the same way as when the test was introduced in the 1960's, excepting the introduction of HEp-2 cells to replace rodent tissue slides as substrate (10;11). F-ANA detects the presence of antibodies to ubiquitous intracellular antigens, resulting in high sensitivity for SLE as many SLE patients have multiple ANA(12). What F-ANA however does not provide is molecular information about the specificity of the antigen(s) recognized by these antibodies and it is the fine specificity of these ANA and their potential clinical correlates that are the matter of interest. In SLE, the specificities of interest are currently represented by the presence of anti-Sm Ab and/or anti-dsDNA Ab (8). While some reports indicate a certain correlation between fluorescence patterns observed in F-ANA and the presence of antibodies against characteristic intracellular antigens, this method is not very reliable and, more importantly, very much operator dependent. Thus, in the case of a positive F-ANA test, all reference laboratories for F-ANA advise or proceed directly to subsequent characterisation of antigen specificity with additional assays.

Given the fact that a positive F-ANA always needs further workup to determine antigen specificity and the fact that a negative F-ANA is only useful in excluding the relatively rare disorder SLE, it may be time to consider alternatives to F-ANA testing. A large body of work has been done in the last decades to define antigen specificity for many of the autoantibodies in the various CTDs and, as a result, specific test methods are now available. While F-ANA has been instrumental in promoting this development it seems to have reached the end of its potential and needs to make room for the more specific tests available for CTDs. Generation shifts are, of course, not easy and making way imparts heartaches, conflicts and uncertainties. Just as the evidence on which F-ANA was introduced as a criterion for SLE was incomplete, we will need to refine our knowledge on the fine specificities of autoantibodies in CTDs. However, if further developments within CTD diagnostics are to continue, a change from F-ANA to specific autoantibody testing seems inevitable. This would allow the F-ANA test to take its well deserved place in medical history.

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Improved Serological Differentiation Between Systemic Lupus and Mixed Connective Tissue Disease Patients using synthetic SmD peptides

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Introduction

Systemic rheumatic diseases are characterized by the occurrence of circulating autoantibodies to defined intracellular targets [reviewed in 1]. Among the earliest identified autoantibodies were those directed to components of U2-U6 small nuclear ribonucleoproteins (snRNPs) known as Sm, which are highly specific for systemic lupus erythematosus (SLE) [2]. Some years later in 1971 anti-U1 RNP antibodies were first described in a patient suffering from SLE [3]. Apart from autoantibodies targeting the Sm-complex, more than 100 different autoantibodies such as anti-dsDNA, anti-proliferating cell nuclear antigen (PCNA), anti-U1-RNP, anti-nucleosome, anti-histone, anti-Ro/SS-A, anti-La/SS-B, anti-ribosomal RNP and anti-phospholipid antibodies have been identified and characterized in sera from SLE patients [4]. Recent studies suggest that SLE- associated antibodies are present before the clinical onset of the disease and thus have high prognostic value [5].

Systemic lupus erythematosus

SLE is an autoimmune disorder which is characterized by chronic inflammatory connective tissue disease. The etiology is still poorly understood, but involves a broad range of factors such as genetic background, immunological and environmental aspects, as well, possibly, as infectious agents. Clinical findings vary greatly. SLE may begin abruptly with fever, simulating acute infection, or may develop insidiously over months or years with episodes of fever and malaise. Vascular headaches, epilepsy, or psychoses may be initial findings. Manifestations referable to any organ system may appear. Articular symptoms, ranging from intermittent arthralgia to acute polyarthritis, occur in approximately 90% of patients and may exist for years before other manifestations appear.

Mixed connective tissue disease

Mixed connective tissue disease (MCTD) is a rheumatic disease syndrome characterized by overlapping clinical features of SLE, systemic sclerosis (SSc), polymyositis (PM) or dermatomyositis (DM), and rheumatoide arthritis (RA). The diagnostic hallmark of MCTD is very high titers of circulating antinuclear antibody to a nuclear ribonucleoprotein antigen. In some patients, the disorder evolves over time into classic SSc, SLE, or even RA. Nevertheless, several features support the suggestion that MCTD is a distinct clinical entity. The typical clinical syndrome is characterized by Raynaud's phenomenon (RP), polyarthralgia or arthritis, swollen hands (dactylitis), inflammatory proximal myopathy, esophageal hypomotility, and pulmonary disease. RP may precede other disease manifestations by years, and frequently the initial findings suggest early SLE, SSc, PM or DM, or RA. Whatever the initial presentation, there is a tendency for more limited disease to progress and become widespread and for transitions in the clinical pattern to occur over time.

Characteristics of anti-Sm antibodies

Anti-Sm reactivity is found in 5-30% of patients with SLE, and this frequency varies depending on the detection system and the ethnicity of the SLE population under investigation [1, 4, 6]. The Sm-antigen is part of the spliceosomal complex that catalyzes the splicing of nuclear pre-mRNA and is composed of at least nine different polypeptides with molecular weights ranging from 9 – 29.5 kDa [B (B1, 28 kDa), B´(B2, 29 kDa), N (B3, 29.5 kDa), D1 (16 kDa), D2 (16.5 kDa), D3 (18 kDa), E (12 kDa), F (11 kDa) and G (9 kDa)] [6]. All of these core proteins, but most frequently the B and D polypeptides, are targets of the anti-Sm autoimmune response [6, 7]. However, since SmBB´ and U1 specific RNPs share the cross-reactive epitope motif PPPGMRPP, SmD is regarded as the most SLE specific Sm-antigen [8]. Within the SmD autoantigen family, reactivity with SmD1/D3 pattern is at least four times more common than SmD1/D2/D3 recognition with a pronounced immunoreactivity to SmD1 [9]. In epitope mapping studies, several linear and conformational epitopes have been mapped on the SmB- and D- proteins [6]. On SmD1 and BB´ the major reactivity was predominantly found in the C-terminal extensions [6]. Follow-up studies and immunization experiments revealed that this motif is consistently the earliest detectable SmBB´ epitope acting as a potential starting point for epitope-spreading events associated with the BB´ molecule and to the SmD- polypeptides [10]. Recently, it has been shown, that the polypeptides D1, D3 and BB´ contain symmetrical dimethylarginine (sDMA) constituting a major autoepitope within the C-terminus of SmD1 and SmD3 [11]. Whether this modified amino acid plays a central role in the development of the SLE-specific B-cell immune response to the Sm particles remains a matter of speculation.

Serological testing for anti-Sm antibodies

Various techniques, in combination with a variety of different antigens, have been proposed for the detection of Sm antibodies: double immunodiffusion, immunoblotting, immunoprecipitation, ELISA, protein microarrays and bead arrays with native antigens from different sources, purified or recombinant proteins, and synthetic peptides [1, 2, 6, 12, 13]. Historically, most anti-Sm antibody tests use Sm antigens purified from a native source containing all Sm polypeptides or even low concentrations of other proteins such as U1 specific RNPs. About ten years ago recombinant SmBB´ from bacteria or insect cells became a favoured antigen of researchers and diagnostic kit manufacturers for the detection of anti-Sm antibodies. However, recombinant SmBB´ and purified Sm antigen containing SmBB´ both bear the disadvantage that they contain the cross-reactive epitope PPPGMRPP, which is present in SmBB´ and in the U1 specific RNPs [8]. Since this epitope is frequently targeted by antibodies in sera from MCTD patients, common anti-Sm antibody assays with purified Sm or recombinant SmBB´ fail to differentiate between SLE and MCTD patients.

In contrast, using anti-Sm assays based on a single peptide derived from the SmD sequence, only a subset of anti-Sm antibodies is detected

[14, 15]. Other Sm autoantibody specificities such as the cross-reactive antibodies recognizing the epitope PPPGMRPP which is shared between SmBB' and U1 specific RNPs are not detected by the SmD peptide based assays. In two recent studies from independent groups it could be elegantly shown that peptide based immunoassays using either a SmD1 or a SmD3 peptide are superior to conventional anti-Sm immunoassays for the detection of anti-Sm antibodies [15, 16]. One of these SmD peptides contains a dimethylarginine residue at position 112 of SmD3 comprising the sequence 108 AARG sDMA GRGMGRGNIF 122 [14, 15].

The correlation between the results of conventional anti-Sm immunoassays with purified Sm antigen from native sources and the peptide assays is relatively poor. Therefore, one might conclude that antibodies targeting SmD derived peptides represent only a minor subpopulation of anti-Sm antibodies [15]. Nevertheless, based on the high sensitivity and specificity and on the observation that antibodies against SmD derived peptides can be used to discriminate MCTD from SLE patients, we conclude that this subpopulation represents an important SLE specific antibody [15].

Peptides as antigens

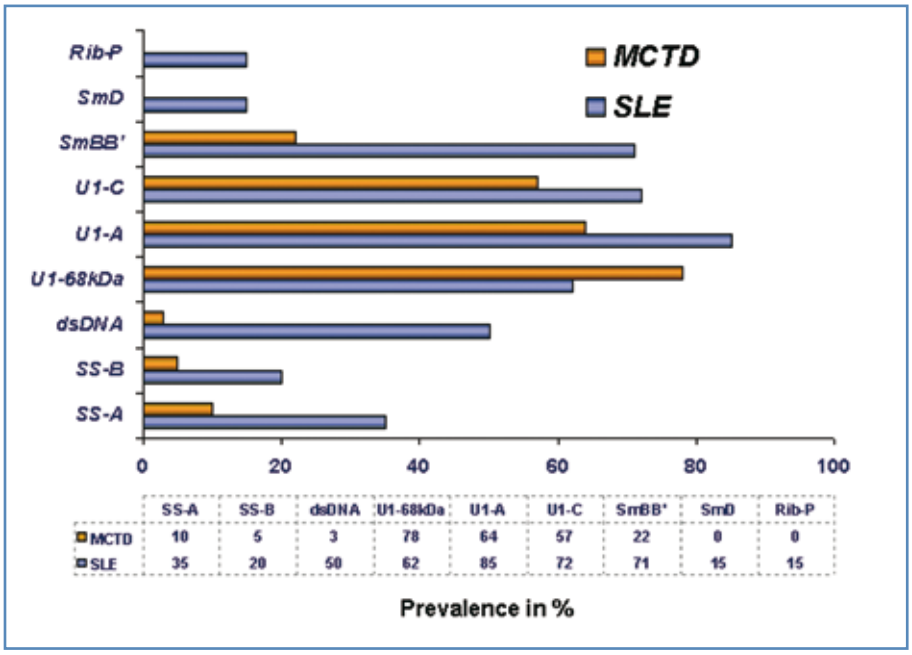
Bruce Merrifield was awarded the Nobel Price in 1985 for the development of chemical peptide synthesis on solid supports (SPPS, solid phase peptide synthesis), a technique, which, ever since, has tremendously advanced research in the fields of chemistry, biochemistry, molecular biology and medicine. Solid phase peptide synthesis starts from a special resin on which the whole peptide sequence is synthesized sequentially by stepwise addition of the different amino acids (linear synthesis). The growing peptide chain remains fixed to the resin until the end of the synthesis. Each single amino acid addition consists of 3 individual reaction steps (deprotection, activation and coupling). After completion of all the amino acid additions, the peptide is present in protected form and still linked to the solid phase. After deprotection of the side chains the raw peptide solution is ready for purification or for the intended use of the peptide.

Quality control of the peptides is usually done by different methods. An analytical high pressure liquid chromatography (HPLC) analysis allows a general evaluation of the purity of the peptide. The HPLC profile of a peptide provides information on its purity, but gives no indication on the peptide sequence. It is therefore very important to check the identity of the peptide by mass spectrometer (MALDI-TOF = Matrix Assisted Laser Desorption Ionization – Time of Flight), which gives the molecular mass of the obtained peptide. Mass spectrometry is the best method to identify the correct peptide. Purified peptides or crude extracts are finally lyophilised, weighed and shipped. The choice of the peptide purity is a very individual matter. From experience, we have found that a purity of 70% might be satisfactory for the development of diagnostic tests. However, in some cases high purity peptides (> 95%) are mandatory to achieve a highly specific assay.

Modification of peptides is of high interest for autoimmune research, diagnostics and therapeutics. The most widespread modifications are biotinylation, fluorescent labels, phosphorylation, disulfide-bridged cyclic peptides, MAP-peptides, branched peptides and peptides containing non-natural amino acids.

Depending on the amino acid sequence, the length and the biochemical properties of the peptide of interest, one of the following coupling strategies can be used to link the peptide to the solid phase of the respective immunoassay (e.g. microtiter plate). Peptides with a length of more than 10 amino acids and an isoelectric point of more than 8 can directly be coated onto microtiter plates. Depending on the structure of the peptide, there is a risk of blocking the epitope when direct coating is used. To increase the absorption properties of synthetic peptides, the peptide must be converted to high-molecular weight products. This can achieved using two strategies. The first possibility is to synthesize the peptide on a special resin, the so-called MAP resin (MAP = multiple antigen peptide). In this approach, 4 to 8 copies of the peptide are synthesized on a polylysine core. After cleavage from the synthesis resin, the resulting MAP construct reaches a molecular weight of about 13-17kDa, which is sufficient for direct coating onto microtiter plates. Alternatively, the peptide of interest can be covalently coupled to so-called carrier proteins such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) or ovalbumin (OVA), which leads to a surface exposure of the peptide and to a higher accessibility of the epitope. Sophisticated surface modifications of microtiter plates, microbeads or microchips allow for the covalent and directed linking of peptides to the respective surface. Similarly, avidin coated surfaces can be used to immobilize biotinylated peptides with high affinity.

Once a synthetic peptide is recognized by a considerable number of sera within a defined cohort of patients with a certain autoimmune disease, it represents an ideal antigenic target for immunoassays because it can easily be produced in high quality and quantity. Furthermore, lower lot to lot variations will be observed since the production is not dependent on the biological variation of native sources of antigens. On the other side of the coin, there are pitfalls to the use of synthetic peptides. False positive results



	SS-A	SS-B	dsDNA	U1-68kDa	U1-A	U1-C	SmBB'	SmD	Rib-P
MCTD	10	5	3	78	64	57	22	0	0
SLE	35	20	50	62	85	72	71	15	15

Figure 1: *Autoantibody profile of SLE and MCTD patients. Antibodies to U1-68kDa, U1-A, U1-C, SmBB', dsDNA, SS-A and SS-B are found in both SLE and MCTD patients. In contrast, anti-SmD and anti-Rib-P antibodies are exclusively present in patients suffering from SLE.*

Given prevalences are based on average values from literature data and on own experiences. Exact values depend on the detection method, on the genetic background of the patients and the make-up of the patient cohorts.

may occasionally occur because the peptides shares amino acid sequences with foreign or self antigens, or because chemical conjugation alters the antigenicity of the peptide.

Nevertheless, today's sophisticated epitope mapping methods will likely lead to the identification of additional peptides, which can be used as specific targets in diagnostic and therapeutic approaches to patient management. This may lead to a new scientific research area with high impact for the development of diagnostic and therapeutic products, in the area of peptide engineering.

Conclusion

Based on various studies, there are strong arguments for the use of synthetic SmD peptides for anti-Sm autoantibody detection. The high purity of synthetic polypeptides will allow for a maximization of both sensitivity and specificity. Moreover, synthetic peptides might be helpful in the standardization of antibody assays. Whether SmD derived peptides become the gold standard for the detection of anti-Sm antibodies remains a matter of further rigorous investigations to access the precise clinical impact of anti-SMP antibodies. It is unlikely that short synthetic peptides will substitute for native or recombinant antigens in all instances since some autoantibodies show a striking preference for conformational epitopes.

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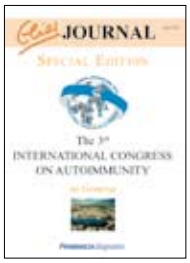

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