The 5th FESCC Continuous Postgraduate Course in Clinical Chemistry

Under the Auspices of IFCC

NEW TRENDS IN CLASSIFICATION, MONITORING AND MANAGEMENT OF AUTOIMMUNE DISEASES

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Table of contents

Editorial

In the past few years, scientists have made some important breakthroughs in understanding the many types of autoimmune disease. These findings are now opening the way to new horizons for diagnosing and monitoring of these disorders. Renowned experts from European countries have participated in this specialized FESCC Course covering the clinical and laboratory aspects of autoimmune diseases. This issue contains the material prepared by these experts especially for the 5th FESCC Course, integrating the knowledge of the authors, experts in different fields, and intending to provide the reader with optimal information.

The contents is divided into three chapters: chapter Basic Concepts covers topics such as New aspects on the immunopathogenesis of autoimmune disease; Genetics of autoimmune disease; Autoimmune aspects of pregnancy and infertility; Apoptosis and autoimmunity; chapter New Approach to Diagnosis covers laboratory standards in the diagnosis and therapy monitoring of autoimmune diseases such as celiac disease, rheumatoid arthritis, vasculitis, multiple sclerosis and SLE; and chapter dedicated to Evidence Based Diagnosis and Quality Assurance deals with Quality assurance and organization of autoantibody laboratory; Statistical management of autoimmune disease data; Guidelines for ANA testing; and Therapeutic potential in autoimmune disease.

We do hope that the contents of this issue will meet the intended goals by presenting the state-of-the-art and contributing to harmonization of the classification, monitoring and management of autoimmune disease.

Elizabeta Topić

1. NEW ASPECTS IN THE IMMUNO-PATHOGENESIS OF AUTOIMMUNITY

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Over the last few decades we are observing a dramatic change in prevalences and incidences of the pattern of major diseases, particularly in Westernized and industrialized countries around the world. On the one hand side, many (acute) infectious diseases are markedly decreasing, including bacterial, viral and fungal infections. On the other side, almost all chronic inflammatory diseases are sharply increasing at the same time. This includes allergies and asthma as well as autoimmune diseases, regardless whether they are belonging to the group of systemic autoimmune disorders or organ-specific diseases. The question arises, whether this is just a coincidence or if this is the result of a cause-effect relationship.

The clinical manifestation and development of the phenotype of autoimmune diseases is strongly dependent on a marked dysregulation on the level of innate and adaptive immune responses. More recent data also indicate that the peripheral nervous system seems to be also involved in triggering the initiation and effector phase of chronic inflammatory conditions. These imbalances in both, the immune and nervous system, are the result of a complex interplay between a genetic disposition and environmental factors. Particularly regarding the development of the immuno-pathogenesis of autoimmune diseases, major advances have been made over the last two decades (Figure 1.1.).

Figure 1.1. *Development of autoimmunity depends on gen-environment interactions*

A major breakthrough in the understanding of the importance of adaptive immunity in this context was the development of the so termed “TH-1/TH-2 Concept” which had been developed about two decades ago. The development of such distinct T-cell subsets has been originally described in the mouse and could be later also extrapolated and proven in the human immune system. The phenotype of these T-cell subpopulations is defined by the cytokine pattern secreted by such T-cells. A leader-cytokine of TH-1 T-cells is interferon γ

(IFN-γ), whereas TH-2 cells are defined by the secretion of IL-4, IL-5, IL-9 and IL-13. These T-cells play an important role in the regulation of normal immune functions (Figure 1.2.).

Figure 1.2. *Normal functions of T-cell effector subsets*

TH-1 T-cell responses are needed to defend many infections, including bacterial, viral, fungal and protozoic infections. These cells also have anti-tumor activities, and regulate the production of IgG and IgM isotypes. In contrast, TH-2 cells are needed to defend helminthic infections, they play an important role in maintenance of successful pregnancy, and they induce isotype-switching towards IgE and IgA (Figure 2).

The next important advancement in this field was the discovery that such T-cell responses are out of balance in many chronic inflammatory diseases. An enhanced or augmented TH-1 response profile was identified in many organ-specific autoimmune diseases, but also in other clinical conditions (Figure 1.3.).

Figure 1.3. *The role of TH-1 and TH-2 T-cells in human disease*

Originally the concept has been put forward that the dysregulation on the level of TH-1 and TH-2 immunity is just a matter of balance between these two distinct subpopulations. However, it now becomes clear that this is not the case under all circumstances. In contrast, the presences of TH-1 or TH-2 T-cell effector responses in diseases are regarded as an imbalanced inflammatory response. This leads to the question: What are the control mechanisms to prevent or inhibit an existing TH-1/TH-2 dysbalance? In previous years it was difficult or even impossible to identify "immuno-suppressive" T-cell responses. This has led to a great conflict among leading immunologist whether such immuno-supressors are existing at all and how they could work possibly. More recently now, due to new technologies and major advancements in the field of T-cell effector responses, this concept of anti-inflammatory T-cell activities are being observed in a new light. The hallmark in this context was the identification of a new T-cell subset producing two important anti-inflammatory cytokines, namely IL-10 and TGF-β. This T-cell subset is now being termed as "regulatory T-cells" (Figure 1.4.). Further advancement in this field indicates that there are several distinct subgroups of regulatory T-cells. Regulatory T-cells have been identified within the CD4, but also the CD8 compartment, and even more recently a subset of NK-T-cells has been shown to possess also regulatory T-cell activities. In general, there are two major sources of regulatory T-cells. One group originates in the thymus. On the other hand, under certain conditions, regular T-cells can also develop in the periphery.

Figure 1.4. *Control mechanism of the TH-1/TH-2 effector response*

The development of pro- as well as anti-inflammatory T-cell subsets is strongly dependent on the instruction by the innate immune system. In this regard, dendritic cells (DCs) play a decisive role. Whereas immature DCs are excellent antigen presenters, it is the job of mature DCs to instruct and activate T-cells. When immature DCs present antigen to naïve T-cells, normally these T-cells become deleted or anergic. This is an important pathway for the development of clinical tolerance. Under normal conditions, activation of T-cells by mature DCs results either in desired and wanted immunity or in autoimmunity.

This depends on the level and strength of cross-reactivity of the presented antigen with self. If the presented antigen does not cross-react with self, immunity results as a consequence of T-cell activation and these T-cells can then develop into normal TH-1 or TH-2 effector T-cells. In contrast, if the presented antigen shows cross-reactivity with self proteins, then the result would be autoimmunity. Dependent on the level of cross-reactivity, these autoimmune responses are either transient or stable. What type of antigens is potentially cross-reactive with self? In this regard, two concepts have been independently developed. On the one hand side, molecular mimicry in the case of an infectious disease seems to be important. On the other hand, bystander activation might be an at least as important mechanism to induce an autoimmune response. Such bystander activation can occur when an immature DC recognizes microbial patterns through their toll-like receptors. When this immature DC presents a self peptide, no further T-cell activation occurs. In contrast, if the immature DC receives additional activation signals through toll-like receptors (viral or microbial components), unwanted activation of such an autoactive T-cell might follow.

This novel concept may explain why autoaggression can be induced and triggered in the presence of microbial antigens.

The development of regulatory T-cells is absolutely necessary in order to control and prevent the development of chronic inflammatory conditions. These T-cells play an important role in the development of “clinical tolerance”. It is well known that the development of clinical tolerance is an active immune mechanism, requiring antigen contact. This process starts already prenatally through the presentation of antigens via the placental barrier to the foetal immune system. However, this is a life-long process which must be maintained at any time. Clinical tolerance is strictly T-cell dependent, and a variety of molecular mechanisms are involved. One major mechanism already starts in the thymus, where potentially autoreactive T-cells are being deleted (Figure 1.5.).

Figure 1.5. *Thymic derived T-cell subsets*

However, this process of central deletion is not complete and allows the escape of some autoreactive T-cells into the peripheral immune system (Figure 1.6.). Here in the periphery, the fate of these T-cells strongly depends on the presence of mature or immature DCs, triggering these cells in a wanted or unwanted fashion.

Figure 1.6. *Characteristics of naturally occurring regulatory T-cells*

In this regard, the presence of regulatory T-cells is absolutely essential. One experiment of *Nature*, where patients lack regulatory T-cells due to mutations in critical genes, illustrates the importance of this T-cell population. These immuno-deficiency syndromes are either termed as the x-linked autoimmunity-allergic dysregulation syndrome (XLAAD) or as the immune dysregulation polyendocrinopathy, enteropathy and x-linked inheritance syndrome (IPEX). In both diseases, patients developed simultaneously severe autoimmune phenotypes, together with allergies.

It will be important for the future to design new modes of immuno-intervention and immuno-prevention, based on this novel concept of immuno-regulation in chronic inflammatory disease. There might be several avenues to strengthen the development of regulatory T-cells and, therefore, the development of “clinical tolerance”. Particularly early in life, the exposure to a variety of microbial compounds seems to be necessary for shaping the tolerance programming. This concept receives currently some support through clinical and experimental studies, investigating the potential of so termed “probiotics”. However, until now it is too early to utilize this knowledge for clinical interference. Major research is currently under way to design and to develop new modes of prevention and treatment of autoimmunity based on this concept.

Literature

1. Shlomchik MJ, Craft JE, Mamula MJ. From T to B and back again: Positive feedback in systemic autoimmune disease. Nature 2001; 1:147-53.
2. Ohashi PS. T-cell signalling and autoimmunity: Moleculare mechanisms of disease. Nature 2002; 2:427-38.
3. Walker LSK, Abbas AK. The enemy within: Keeping self-reactive T cells at bay in the periphery. Nature 2002; 2:11-9.
4. von Herrath MG, Harrison LC. Antigen-induced regulatory T cells in autoimmunity. Nature 2003;3:223-32.
5. Kyewski B, Derbinski Jens. Self-representation in the thymus: An extended view. Nature 2004; 4: 688-98.

2. GENETICS OF AUTOIMMUNE DISEASE

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Autoimmune diseases are common conditions which affect up to 10% of the general population. The reasons why individuals develop an autoimmune disease are largely unknown. It seems to develop in genetically susceptible individuals and the course of the disease can be influenced in a permissive or in a protective way.

To study the genetic risk of getting an autoimmune disease several approaches have been used. The oldest and most simple way is the simple description of the same autoimmune disease occuring in different members of the same family. These multicase families with autoimmunity suggest a genetic modified etiology as well as the possibility of shared environmental factors in the pathogenesis of these diseases. Other approaches are concordance studies in monocygotic and dizygotic twins. Concordance rates for autoimmune diseases in monocygotic twins are between 30% and 70% but not 100% (Table 2.1.) indicating that these diseases are a result of genetic and environmental factors.

Table 2.1. *Concordance rates in monozygotic and dizygotic twins*

In addition that these observations of finding the same autoimmune deseases within families also a tendency for multiple different autoimmune diseases can be seen with increased frequency among first and second degree relatives of a person with a given autoimmune disease. These observations imply the possibility that common genes predispose to different forms of autoimmunity. There are two ways in humans which have been used to identify susceptibility genes of common diseases either by testing hypothesized candidate genes or by whole genome scanning methods. Candidate genes are genes located in a chromosome region suspected of being involved in a disease. Candidate gene studies using cohort comparisons between affected patients and racially and geographically matched healthy controls have shown that the major histocompatibility complex (MHC) region on chomosome 6 has the strongest association with most immune-mediated diseases. Also other polymorphic genetic loci including genes encoding cytokines and cytokine receptors, T-cell receptors, immunoglobulins, Fc receptors and autoantigens have been identified as risk factors for

various autoimmune diseases but their statistical association with disease has been found to be weaker than those of the MHC complex. Nevertheless these other genetic loci are involved in autoimmune diseases as secondary risk factors.

The HLA region on chromosome 6p21 can be split into three different parts called class I, class II and class III. The class I region encodes HLA-A, HLA-B and HLA-C molecules which are expressed on the cell surface of nucleated cells involved in the presentation of endogenous antigens to CD8+ cytotoxic T (Tc) cells. The class II region encodes many membrane-bound proteins expressed on the cell surfaces of B-lymphocytes, macrophages, dendritic cells and activated T lymphocytes, which are involved in the processing and presentation of exogenous antigens to CD4+ T-helper (Th) cells. The class III region is located between the class I and class II regions and contains genes encoding components of the complement region (C2 and C4), the heat shock protein (HSP70) and the tumour necrosis factors (TNF).

HLA class I antigens have been associated with psoriasis. According to the age of onset psoriasis has been subdivided into a familial early age (< 40 years) of onset form (type I) and a sporadic late onset form with no family history (type II). Type I psoriasis has a high association to genes of the MHC complex most strongly with HLA-Cw6 and HLA-B57. HLA-Cw6 seems to influence the age of disease onset with concordance rates of 80% in developing the disease before 20 years of age. Up to 30% of psoriasis patients develop psoriatic arthritis (PsA) making PsA to one of the most often spondylarthropathies. PsA patients with psoriasis type I show similar HLA assiciations as type I patients without arthritis but different from patients with arthritis and late onset disease. HLA-B27 has been related to spine involvement and HLA-B39 to polyarthritic disease in PsA patients.

HLA-B27 is found in a healthy white population in about 8% but in patients with spondylarthropathies with increased rates (ankylosing spondylitis 95% of patients, reactive arthritis 70%, psoriatic arthritis 60%, psoriatic arthritis with peripheral arthritis 25%, spondylitis with inflammatory bowel disease 70%, acute anterior uveitis without any other stigmata of spondyloarthritis 50%). The exact mechanism underlying the effect of HLA-B27 on disease susceptibility is still unknown. Interestingly no association of HLA-B27 is seen in patients with spondylarthritis in Africa.

HLA class II region contributes to most autoimmune dieases. The underlying mechanisms remain unknown but seem to be different for each disease.

In insulin-dependent diabetes mellitus (IDDM) about 34% of familial clustering is due to the MHC class II region. HLA alleles associated with diabetes susceptbility include HLA-DR3 and HLA-DR4 wheras others are associated with diease protection like HLA-DR2. On the other hand HLA-DR2 seems to predispose to multiple sclerosis (MS). The protective nature of HLA-DR2 in IDDM and the predisposing nature in MS could be the reason why it is rare to see clustering of MS in IDDM and vice versa. In MS the specific genes with increased risk are the HLA-DR and the HLA-DQ genes, the HLA-DR15 haplotype in Caucasians and other DRs in ethnically more distant populations.

HLA-DR4 phenotype is regarded as a genetic determinant commonly associated with rheumatoid arthritis (RA). The major susceptibility alleles associated with RA are the HLA-DR4 alleles DRB1*0401 and DRB1*0404. Caucasians with DRB1*0401/0404 seem to have a higher risk of a more severe form of RA.

HLA-DR3 appears to be a general autoimmune haplotype not only associated with IDDM but also with systemic lupus erythematodes (SLE), Graves' disease, autoimmune hypothyroidism an Addison's disease. Among all immunogenes tested in complex and autoimmune liver diseases strongest disease associstions were found with the MHC HLA class II genes DR and DQ.

The HLA class III region contains many genes encoding proteins which are unrelated to cell-mediated immunity but modulate or regulate immune responses in some way, including tumour necrosis factor, heat shock proteins and complement proteins (C2, C4). The complement genes C2 and C4 have shown to be associated with SLE with an incidence of 75% of C4 homozygous subjects and 33% of C2 homozygous subjects developing SLE. The hierarchy of susceptibility amongst these components is C1q>C4>C2 in disease risk order.

Also other genes beside the HLA genes seem to be involved in susceptibility for autoimmune diseases. Organ specific autoimmune disease susceptibility loci are for example the insulin gene (INS) region on chromosome 11p15 or the cytotoxic T-lymphocyte-associated-4 (CTLA-4) gene on chromosome 2q33. CTLA-4 was first identified as a candidate gene in Graves' disease but is an equally strong candidate for other T-cell mediated autoimmune diseases like IDDM. Non-organ specific autoimmune disease susceptibility loci are for example genes for proinflammatory cytokines like TNF or IL-1.

Genetic susceptibility to the development of autoimmune disease is a complex subject with many different genes and their products interacting with each other and interacting with external stimuli. Certain gene regions, especially HLA, are likely to cause susceptibility to more than one autoimmune disease and might explain the clustering of diseases within the same families and individuals.

Literature

1. Fathman CG, Soares L, Chan SM, Utz PJ. An array of possibilities for the study of autoimmunity. Nature 2005; 435:605-11.
2. Reveille JD, Frank MD, Arnett FC. Spondylarthritis: update on pathogenesis and management. Am J Med 2005; 118:592-603.
3. Rioux JD, Abbas AK. Paths to uderstanding the genetic basis of autoimmune disease. Nature 2005; 435:584-9.
4. Simmonds MJ, Gough SCL. Genetic insights into disease mechanisms of autoimmunity. Brit Med Bull 2005; 71:93-113.
5. Shamim EA, Rider LG, Miller FW. Update on the genetics of the iodiopathic inflammatory myopathies. Curr Op Rheumatol 2000; 12:482-91.

3. AUTOIMMUNE ASPECTS OF PREGNANCY AND INFERTILITY

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3.1 Background

Autoimmune diseases affect more women than man. For example, it has been estimated that 75 % of roughly 8.5 million people who suffer of an autoimmune disorder in the United States are female. The precise reasons for this gender bias are unclear, but sex hormones and / or sex hormone related genes my modulate susceptibility.

Distinct immune environments in males and females underlie many of the sex differences in autoimmunity. These environments are established by the cytokines released by immune cells, particularly T helper (T_H) lymphocytes. Females are more likely to develop a T_H1 response (dominated by interleukin-2 (IL-2), interferon-γ (IFN-γ), and lymphotoxin) after challenge with an infectious agent or antigen, except during pregnancy when a T_H2 environment prevails (dominated by IL-4, IL-5, IL-6, IL-10 and TGF-β). Furthermore, the degree of immune response also differs between men and women. As androgens seem to be primarily suppressive on cellular and humoral immunity, immune responses tend to be more vigorous in females, resulting in greater antibody production and increased cell-mediated immunity after immunisation. There are several possible ways in which sex hormones could affect the immune system. They may modulate T cell receptor signaling, expression of activation molecules on T lymphocytes and antigen-presenting cells, transcription or translation of cytokine genes, or lymphocyte homing.

3.2 Effects of pregnancy on the course of autoimmune disorders

Pregnancy in healthy women does not seem to increase the prevalence of autoantibodies in comparison with non-pregnant control groups, but may differently affect the clinical course of several autoimmune disorders, with important consequences for both mother and offspring. In multiple sclerosis and rheumatoid arthritis, there is a decrease in disease severity during the 9 months of gestation, with a return to pre-pregnancy levels after birth. This is in contrast to lupus where the disease may worsen during pregnancy. Thus, as the particular hormone environment during pregnancy favours a T_H2 response, the progression of the T_H1 immune response associated with multiple sclerosis and rheumatoid arthritis may be halted. In contrast, pregnancy may further enhance the ongoing T_H2 (antibody-promoting) response associated with SLE.

The following part of the presentation will focus on common autoimmune disorders that can be regarded as typical examples for clinically relevant autoimmune aspects of pregnancy and infertility.

3.2.1 Rheumatoid arthritis

Pregnancy is associated with improvement in the clinical signs and symptoms of rheumatoid arthritis in more than 70% of patients. Maternal-fetal disparity in alleles of HLA-DRbeta1, DQalpha, and DQbeta has been reported to be associated with pregnancies characterised by remission or improvement, possibly by induction of maternal-regulatory T cells, or by affecting the maternal T cell receptor repertoire via fetal presentation of associated peptides.

3.2.2 Systemic lupus erythematosus (SLE)

The course of SLE is more variable. Whether flare rates increase during or after pregnancy is unsettled, since individual patient series vary in the characteristics of patients accepted for study and in definitions of flare. Despite a high overall flare rate in some series approaching 60%, recorded flares were usually not severe. More recent prospective studies indicate that pregnancy is safe for the majority of mothers - even with lupus nephritis - if pregnancy is planned when SLE is quiescent. Scoring systems for SLE related disease activity have been adapted as diagnostic tools for lupus flares during pregnancy and the puerperium. Pregnant lupus patients seem to be susceptible to pre-eclampsia, especially if they suffer lupus nephritis, and to steroid-induced hypertension and hyperglycemia.

Oral contraceptives containing oestrogens and hormone replacement therapy are generally not prescribed for women with systemic lupus erythematosus (SLE). The concern regarding estrogens is based on the greater incidence of SLE in women, abnormalities of oestrogen metabolism, murine models of lupus, several anecdotes of patients having disease flares while receiving hormones, and one retrospective study in patients with pre-existing renal disease. A 12-months hormone replacement therapy was recently shown to be associated with a small risk of increasing the natural flare rate (relative risk 1.34, $p = 0.01$), but most of the flares were mild to moderate, and hormone replacement did not significantly increase the risk for severe flares compared to placebo.

3.2.3 Scleroderma

Only limited data are available regarding the incidence or outcome for either the mother with scleroderma or her fetus. The extent of diffuse skin disease and systemic involvement, particularly pulmonary, cardiac and renal, may be more important than the duration of the disease; limited disease carries a better prognosis for the mother and fetus.

3.3 Effects of maternal autoimmune disorders on the offspring

Transplacental transfer of autoantibodies is common, and autoantibodies can be readily demonstrated in newborn serum. Only a small proportion of infants with circulating autoantibodies exhibit clinical symptoms. The transient neonatal manifestations of maternal autoimmune disease disappear over a time course consistent with the catabolism of IgG, providing no permanent damage occurs. Thus the pathogenic role of transferred autoantibodies seems well established. However, maternal-autoantibody-mediated tissue damage appears to depend on factors other than the mere passage of the antibody to the fetal compartment.

3.3.1 SLE

The rate of loss in SLE pregnancies has decreased from a mean as high as 43 % before 1975 to 17 % and 14 % in two recent series and was found to be similar to the general US population. However, the rate of preterm delivery in mothers with SLE was still around 33 % and thus nearly the triple of what would be expected. Furthermore, fewer life births occurred among women with high-activity lupus compared to those with low-activity SLE, with high disease activity during the first and second trimesters being associated with a 3-fold increase in pregnancy loss. Especially, the survival of the fetus is strongly in doubt when cyclophosphamide is required to treat lupus in the mother. Therefore, the old dogma, that women with SLE are advised to consider pregnancy only when disease is stable, seems still to be valid. Maternal SLE does not seem to impair intelligence levels of the children, but learning disabilities have been described especially in male offsprings, and may be associated to maternal anti-Ro/La antibodies.

3.3.2 Neonatal lupus syndromes (NLS)

The neonatal lupus syndromes (NLS), while quite rare, carry significant mortality and morbidity in cases of cardiac manifestations. Anti-SSA/Ro-SSB/La antibodies are detected in > 85% of mothers whose fetuses are identified with congenital heart block in a structurally normal heart. However, the risk for a woman with the candidate antibodies to have a child with congenital heart block was described to be at or below 2 %. While the precise pathogenic mechanism of antibody-mediated injury remains unknown, it is clear that the antibodies alone are insufficient to cause disease and fetal factors are likely contributory, including apoptosis of cardiocytes, surface translocation of Ro and La antigens, binding of maternal autoantibodies, and a scarring process that involves TGFbeta and cardiac myofibroflast. The spectrum of cardiac abnormalities continues to expand, with varying degrees of block identified in utero and reports of late onset cardiomyopathy.

Moreover, there is now clear documentation that incomplete blocks can progress postnatally, despite the clearance of the maternal antibodies from the neonatal circulation. Furthermore, cutaneous, hematologic, hepatic abnormalities and serositis have been described, but are usually transient. Mothers of affected infants are often asymptomatic, and when symptomatic, the clinical features are frequently characteristic of Sjögren´s syndrome.

3.3.3 Immunosuppressive drugs during pregnancy

The management of pregnancy in patients with autoimmune disorders includes the treatment of disease flares, using drugs effective but safe for the fetus. Corticosteroidsare routinely used to control maternal disease. Some immunosuppressive drugs such as azathioprine may also be regarded as relatively safe, whereas others such as cyclophosphamide and methotrexate are clearly contraindicated. The last 10-year experience shows that fetal exposure to antimalarial drugs should not be regarded as an important risk factor for gestational nor neonatal complications. However, information about long-term outcome of children exposed to immunosuppressive drugs "in utero" are still lacking and more efforts are needed in this research area.

3.4 Autoimmune aspects of infertility

Both anti-phospholipid and anti-thyroid antibodies have been linked to infertility and pregnancy loss. The *anti-phospholipid syndrome* (APS) is a non-inflammatory disease characterised by the presence of anti-phospholipid antibodies in the plasma of patients with venous or arterial thrombosis or obstetric complications such as recurrent abortions and miscarriage. APS is usually diagnosed in the setting of maternal SLE, but may present as a primary syndrome. An overwhelming activation of complement triggered by antibodies deposited in the placenta seems to be pathogenetically important. Recent data indicate that only a subpopulation of the heterogeneous population of anti-phospholipid antibodies is pathogenic, antibodies against β2-glycoprotein I being especially important. In patients who fulfil criteria for APS, recent papers advocate combined treatment with aspirin (75-100 mg/d) and low molecular weight heparin, rendering obstetric APS to a treatable condition in most patients.

The association between *thyroid autoimmunity* and adverse fetal outcome has been described repetitively and was recently confirmed in a meta-analysis, that found a clear association between the presence of anti-thyroid autoantibodies and miscarriage in case control (odds ratio 2.7; 95 % CI 2.20 - 3.40) and longitudinal studies (odds ratio 2.3; 95 % CI 1.80 - 2.95). In a study investigating anti-thyroid antibodies and anti-phospholipid-antibodies in women with recurrent spontaneous abortions, anti-thyroid antibodies were found in 27 % of patients and were associated with a significantly lower percentage of spontaneous pregnancies and life births when compared with women who were tested positive for anti-phospholipid antibodies and negative for anti-thyroid antibodies. The underlying pathogenetic mechanisms are unclear.

Literature

1. Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol 1997; 84:223-43.
2. Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. Science 1999; 283:1277-8.
3. Cutolo M, Wilder RL. Different roles for androgens and estrogens in the susceptibility to autoimmune rheumatic diseases. Rheum Dis Clin North Am 2000; 26:825-39.
4. Mavridis AK, Ming LX, Hatzipetrou P, Lentzaris G, Papanikolaou NG, Tzioufas AG, Moutsopoulos HM. Prevalence of non-organ-specific autoantibodies in pregnant and non-pregnant healthy women. Lupus 1992; 1:141-4.
5. Buyon JP. The effects of pregnancy on autoimmune diseases. J Leukoc Biol 1998; 63:281-7.
6. Moroni G, Ponticelli C. Pregnancy after lupus nephritis. Lupus 2005; 14:89-94.
7. Ruiz-Irastorza G, Khamashta MA, Gordon C, Lockshin MD, Johns KR, Sammaritano L, Hughes GR. Measuring systemic lupus erythematosus activity during pregnancy: validation of the lupus activity index in pregnancy scale. Arthritis Rheum 2004; 51:78-82.
8. Lockshin MD, Sammaritano LR. Lupus pregnancy. Autoimmunity 2003; 36:33-40.
9. Buyon JP, Petri MA, Kim MY, Kalunian KC, Grossman J, Hahn BH, Merrill JT, Sammaritano L, Lockshin M, Alarcon GS, Manzi S, Belmont HM, Askanase AD, Sigler L, Dooley MA, Von Feldt J, McCune WJ, Friedman A, Wachs J, Cronin M, Hearth-Holmes M, Tan M, Licciardi F.

The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: a randomized trial. Ann Intern Med 2005; 142:953-62.

10. Giacoia GP. Transplacentally transmitted autoimmune disorders of the fetus and newborn: pathogenic considerations. South Med J 1992; 85:139-45.
11. Clark CA, Spitzer KA, Laskin CA. Decrease in pregnancy loss rates in patients with systemic lupus erythematosus over a 40-year period. J Rheumatol 2005; 32:1709-12.
12. Clowse ME, Magder LS, Witter F, Petri M. The impact of increased lupus activity on obstetric outcomes. Arthritis Rheum 2005; 52:514-21.
13. Clowse ME, Magder L, Petri M. Cyclophosphamide for lupus during pregnancy. Lupus 2005;14:593-7.
14. Buyon JP. Dispelling the preconceived notion that lupus pregnancies result in poor outcomes. J Rheumatol 2005; 32:1641-2.
15. Neri F, Chimini L, Bonomi F, Filippini E, Motta M, Faden D, Lojacono A, Rebaioli CB, Frassi M, Danieli E, Tincani A. Neuropsychological development of children born to patients with systemic lupus erythematosus. Lupus 2004;13:805-11.
16. Ross G, Sammaritano L, Nass R, Lockshin M. Effects of mothers' autoimmune disease during pregnancy on learning disabilities and hand preference in their children. Arch Pediatr Adolesc Med 2003; 157:397-402.
17. Buyon JP, Rupel A, Clancy RM: Neonatal lupus syndromes. Lupus 2004;13:705-12.
18. Boh EE. Neonatal lupus erythematosus. Clin Dermatol 2004; 22:125-8.
19. Clancy RM, Backer CB, Yin X, Chang MW, Cohen SR, Lee LA, Buyon JP. Genetic association of cutaneous neonatal lupus with HLA class II and tumor necrosis factor alpha: implications for pathogenesis. Arthritis Rheum 2004; 50:2598-603.
20. Lockshin MD, Sammaritano LR. Corticosteroids during pregnancy. Scand J Rheumatol Suppl 1998; 107:136-8.
21. Tincani A, Rebaioli CB, Frassi M, Taglietti M, Gorla R, Cavazzana I, Faden D, Taddei F, Lojacono A, Motta M, Trepidi L, Meroni P, Cimaz R, Ghirardello A, Doria A, Pisoni MP, Muscara M, Brucato A. Pregnancy and autoimmunity: Maternal treatment and maternal disease influence on pregnancy outcome. Autoimmun Rev 2005; 4:423-8.
22. de Groot PG, Derksen RH. Antiphospholipid antibodies: update on detection, pathophysiology, and treatment. Curr Opin Hematol 2004; 11:165-9.
23. Girardi G, Salmon JB. The role of complement in pregnancy and fetal loss. Autoimmunity 2003; 36:19-26.
24. de Groot PG, Derksen RH. The antiphospholipid syndrome: clinical characteristics, laboratory features and pathogenesis. Curr Opin Infect Dis 2005; 18:205-10.
25. Derksen RH, Khamashta MA, Branch DW. Management of the obstetric antiphospholipid syndrome. Arthritis Rheum 2004; 50:1028-39.
26. Prummel MF, Wiersinga WM. Thyroid autoimmunity and miscarriage. Eur J Endocrinol 2004;150:751-5.
27. De Carolis C, Greco E, Guarino MD, Perricone C, Dal Lago A, Giacomelli R, Fontana L, Perricone R. Anti-thyroid antibodies and antiphospholipid syndrome: evidence of reduced fecundity and of poor pregnancy outcome in recurrent spontaneous aborters. Am J Reprod Immunol 2004; 52:263-6.

4. APOPTOSIS AND AUTOIMMUNITY

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4.1 Introduction

Apoptosis and autoimmunity are two phenomena, which share essential similarities in destroying cells. However, apoptosis is a normal process, while autoimmunity is not. Apoptosis could, at least in some conditions, cause autoimmunity. The failure to appropriately achieve programmed cell death and to clear apoptotic cell fragments is discussed as a key pathogenetic factor leading to autoimmunity.

4.2 Cell death

The term “apoptosis” (from Greek apo = from and ptosis = falling) was first mentioned in 1972 in the article of John Kerr and co-workers (Br J Cancer 1972;26:239-57) to distinguish between developmental cell death and necrosis (from Greek nekros = dead) as unprogrammed death of cells due to injury, infection, cancer, infarction or inflammation. Apoptosis is almost invisible in tissue. In contrast, fibrinoid necrosis, which is caused by immune-mediated vascular damage, is marked by deposition of fibrin-like proteinaceous material in arterial walls seen by microscope.

Apoptosis is a process characterized by the plasma membrane blebbing, cytoplasmic and organelle concentration and shrinkage, nuclear chromatin condensation and DNA cleavage at certain internucleosomal sites, producing characteristic 180-200 base pairs fragments. Finally, generated cellular fragments or apoptotic bodies become rapidly ingested by macrophages or neighboring cells. Although cells undergoing apoptosis show distinct morphological characteristics, their rapid clearance generally results in a very small number of cells that can be categorized as apoptotic at any given time. The highly efficient elimination of apoptotic cells occurs fast and without tissue damage or inflammation. Apoptosis is known as programmed cell death, but the linkage between apoptosis and programmed cell death, evolutionarily conserved process was made in early nineties, more than hundred years after Flemming’s description of the naturally occurring form of cell death, then called “chromatolysis” (Archiv für Anatomie und Physiologie 1885;221-4). Nowadays two forms of physiological or programmed cell death are distinguished: apoptotic or type I and autophagic or type II. Autophagic cell death includes degradation of the Golgi apparatus, polyribosomes, and endoplasmic reticulum, which precede nuclear destruction. Intermediate filaments and microfilaments are largely preserved; presumably the cytoskeleton is required for autophagocytosis. Vacuolization is a common feature.

However, autophagic and apoptotic programmed cell death should not be considered as mutually exclusive phenomena. Rather, they appear to reflect a high degree of flexibility in a cell's response to changes in environmental conditions, both physiological and pathological.

4.3 The purpose of apoptosis

Apoptosis as the main type of a programmed cell death is a process of deliberate suicide by an unwanted cell in a multicellular organism: but apoptosis is not the question of one or a few cells. The amount of cells undergoing apoptosis during the life of a multicellular organism is surprisingly large. In a healthy adult human, billions of cells die in the bone marrow and intestine every hour. In the whole body, more than 50 billion cells die each day due to apoptosis. The mass of yearly destructed cells is equal to an individual's body weight. Apoptosis is important in all stages of life: During embryonic development, apoptosis eliminates cells when the structure they formed is no longer needed: disjointing the “fingers” in a developing vertebrate embryo is the result of apoptotic death of intermediate cells. Neonatal waves of apoptosis had been demonstrated for pancreatic beta-cells due to remodeling of tissue, and increased neonatal apoptosis was found in the kidney, cardiac myocytes, male germ cells and adrenal cortex. In the developing nervous system apoptosis adjusts the number of nerve cells to match the number of target cells that require inervation. Apoptosis play a role also in the negative selection of thymocytes and lymphocytes: it has been estimated that as many as 95% of the T lymphocytes produced are eliminated by apoptosis, presumably because they recognize self-antigens. In the adult organism, the number of cells within an organ or tissue has to be constant within a certain range. Tight coupling of cell death and cell multiplication ensures in many tissues a constant, controlled flux of fresh cells, which are crucial to the preservation and optimal functioning of the organism. Blood, skin, and gut cells are constantly renewed by their respective progenitor cells. A proper turnover of cells is maintained by balancing proliferation with cell death as a part of the homeostasis. Some authors have suggested “homeodynamics” as a more correct and expressive term (Damasio A. The Feeling of What Happens, Harcourt Brace & Co1999, New York p. 141).

Apoptosis has a special role in the vertebrate hemopoietic system: billions of neutrophils die in this way each day in an adult human. The vast majority is perfectly healthy at the time of suicide. Even more, they die in the bone marrow without ever functioning. This fruitless process of production and destruction presumably serves to maintain a reserve supply of cells that can be quickly mobilized to fight infection whenever it flares up. Similar situation is in the immune system, where rapid cell division is required during infections, but cell growth needs to be limited and terminated after clearance of the microbe in order to prevent unregulated proliferation with the consequence of leukemia or lymphoma.

“Compared with the life of the organism, the lives of cells are cheap” (Alberts B, et al. Molecular biology of the cell. Garland Science, 2002).

4.4 Basic molecular mechanisms of apoptosis

The “decision” to activate a suicidal process is made on the basis of intrinsic or extrinsic apoptotic messages. Intrinsic inducers come from mitochondria or nucleus. Extrinsic inducers are ligands (cytokines) for death receptors on the cell surface. But apoptosis can be induced also by lack of signal molecules produced by other cells, which normally work in combinations to regulate the behavior of the cell. An individual cell requires multiple signals to survive; otherwise it will undergo a programmed cell death.

Irrespective of the initial signal, the core role belongs to a family of proteases that have cysteine at their active site and cleave target proteins at a specific aspartic site. They are called

caspases. Since activation of caspases and their cleavage are irreversible, the entrance into apoptosis is in an all-or-none mode, without a possibility to turn back.

Caspases are synthesized in inactive form as procaspases. Each caspase consists of a structurally related molecule with prodomain, a large subunit and a small subunit. The latter two combine to form tetramers after cleavage. When activated, they cleave the aspartic moiety of another procaspases, make them active. Currently 13 different caspases have been described, 11 of them were found in humans. Based on their function and substrate specificity they can be classified into three groups: a/ upstream enzymes, which amplify death signal; b/ CED-3-like caspases (CED = cell death, product of gene ced involved in apoptosis found in Caenorhabditis elegans), which rapidly cleave structural and vital components, and c/ ICE-like caspases (ICE = interleukine-1 beta-converting enzyme), which may be involved in inflammation rather than death.

The leader position among upstream enzymes belongs to initiator procaspases-8. When they are pushed together by adapter proteins, they cleave themselves to active initiator caspases-8. In the enzymatic cascade, caspases' activity is amplified until key reactions are reached:

- Cleavage of a cytoskeleton, making cell circular and less rigid
- Cleavage of nuclear pore proteins, making nuclear envelope more permeable
- Cleavage of nuclear lamins, causing irreversible breakdown of nuclear lamina
- Cleavage of a protein that normally holds a DNase in inactive form
- Cleavage of poly-(ADP-ribose)-polymerase
- Degradation of chromatin and its packaging proteins

4.5 Regulation of the cell death program

Apoptosis is not a result of the disbalance between pro- and anti- apoptotic stimuli. The most important regulatory mechanisms in mammalians are: death receptors, caspases, mitochondria, the Bcl-2 family of proto-oncogenes and tumor-suppressor gene p53.

The main death receptors (DR) are CD95 (Fas), CD120a (TNF-RI), DR3, DR4, DR5 and DR6, which are responsive to cytokines belonging to the tumor necrosis factor family (TNF-alfa, lymphotoxin, FasL, ApoL3). The binding of a ligand provokes trimerization of the receptor and recruitment of intracellular adaptor proteins that aggregate initiator procaspase-8. In contrast to FasL, TNF-alfa mediated signaling may stimulate cell proliferation through the nuclear factor kappa beta pathway, depending on the cell type, the receptor (CD120a or CD120b) and other regulators. The link between apoptosis and TNF activity shows why abnormal production of TNF plays an important role in several autoimmune diseases (e.g. rheumatoid arthritis, diabetes mellitus, multiple sclerosis).

Mitochondria have a fundamental position in executing apoptosis induced by intracellular signals. When cells are stressed due to physical stimulus (UV or gama radiation), chemical stimulus (genotoxic or cytotoxic drugs, metabolic poisons), or an altered conditions (detachment of cells from the extracellular matrix, cytokine- and growth factor-deprivation) mitochondria release pro-apoptotic proteins, including cytochrome c. It binds to an adapter protein Apaf-1, thus activating procaspase-9 and enzymatic cascade. Additional mitochondrial activities leading to apoptosis are the disruption of electron transport and modification of cellular reduction-oxidation potential.

The most important proto-oncogenes involved in maintaining cell survival and proliferation are those belonging to the Bcl-2 family. Its gene products have different functions: Bcl-2 by itself and Bcl-XL are inhibitors of apoptosis, while Bad, Bax, Bak, and Bid are promoters. Protein Bad binds and inactivate inhibitors of apoptosis Bcl-2 and Bcl-XL. Bid is activator of Bax and Bak, which are initiators of apoptosis through stimulating cytochrome c release from mitochondria.

Among tumor-suppressor genes, p53 is the most important. In response to DNA damage, the product p53 can induce apoptosis through the activation of gene encoding cytochrome c releasing proteins.

Inhibitors of apoptosis family (IAP) inhibit apoptosis by deterrence of activity of some caspases and by prevention of activation of some procaspases.

Some other modifiers of apoptosis have been reported. Nitric oxide may increase the mitochondrial membrane potential and may chemically modify cytochrome c, both leading to promote the release of cytochrome c. On the other hand, nitric oxide may decrease the activity of caspases by induction of their S-nitrosylation. High levels of reactive oxygen species promote lipid peroxidation, macromolecular damage and necrotic cell death, while lower levels of reactive oxygen species promote changes in cell signaling that result in apoptosis.

4.6 Apoptosis as causal factor for autoimmunity

Apoptosis is a process “where too much or too little can lead to autoimmunity” (Grodzitsky T, Elkon KB. Mt Sinai J Med 2002;69:208-19). Indeed, the capacity for unrestricted self-renewal is a dangerous property for any cell to possess, and many cases of leukemia occur through mutations that confer this capacity. On the other hand, too much apoptosis of beta isle cells of the pancreas is implicated in the pathogenesis of diabetes mellitus.

Activation of naive T cells by antigen results in the massive expansion of antigen specific T cells. The majority of T cells die due to apoptosis, while a small number survive and differentiate into memory cells. This cell destiny is crucial for our understanding of how autoimmunity is evaded. Some autoreactive cells may escape natural apoptosis and represent continuous threat with potential autoimmune response. But also apoptotic cells do not disappear after phagocytosis. Ingredients of the apoptotic blebs indeed survive the intracellular processing and are recycled to the membrane of the phagocyte. Massive apoptosis overloading the phagocytic capacity may trigger an autoimmune reaction through presentation of nucleosomes to the immune system. Even after death cell components keep being perceptible to the immune system. Natural antibodies with activity to apoptotic blebs, or the first waves of autoantibodies, which bind with relative low affinity cell debris (noticeable also after infection) may increase depletion efficiency via opsonisation; but extensive overload with apoptotic corps, extensive presentation of nucleosomes to lymphocytes and excessive amount of autoreactive antibodies could disbalance the immune/apoptotic homeostasis and contribute to the pathogenesis of systemic and organ specific autoimmune disease. Apoptosis has traditionally been thought of as a non-inflammatory process, which does not induce an immune response. However, recent studies indicate that apoptotic cells can be involved in (auto)immune processes. They can display autoreactive antigen in their surface blebs, they can activate dendritic cells and they can induce the formation of autoantibodies.

4.7 Apoptosis and autoimmune diseases

In systemic autoimmune diseases, especially in systemic lupus erythematosus, autoantibodies against a palette of intra-cellular antigens are found. It has been suggested that humoral response may be driven by the products of apoptotic cells with the central importance of the nucleosomes as potential (auto)antigens. It was shown that after apoptosis, induced in keratinocytes following UV light exposure, the cells express on their surface (in blebs) several intracellular antigens such as Ro, La, ribosomes and nucleosomes; but an increased number of nuclear antigens per se is not sufficient to induce lupus-like autoimmune response. Autoreactive B and T cells, escaped from natural apoptosis might represent additional necessary condition. Caspase activity in apoptotic cells may lead to presentation of cryptic epitopes or neoepitopes to which the immune system is not tolerant: modified antigens (phosphorilated, citrulinated, acetylated) may lead to an increased immunogenicity. Direct access to the cytosol of antigens derived from the processing of engulfed apoptotic cell corpses has been reported in activated phagocytes.

This event results in proteosome-dependent processing of apoptotic cell-associated antigens, translocation into the endoplasmatic reticulum and loading into MHC class I molecules. Antigens, contained in theengulfed apoptotic remnants may use both MHC class I and class II molecule pathways as well as other less-conventional antigen presentation pathway. Observed decreased NF-κB (p65) expression in lupus patients may be linked with inappropriate proteasome-dependent processing, promoting autoimmune response, especially when dendritic cells are involved in phagocytosis of apoptotic cells. Communication among uncleared apoptotic cells and interferon-α excreted dendritic cells plays an important role in the pathogenesis of disease. Increased CD95, CD95L, and CD40L expression, decreased TGFβ secretion, defects in phagocytosis are fragments of our knowledge, which suggest a strong involvement of the apoptotic processes in the pathogenesis of systemic lupus erythematosus.

Antiphospholipid syndrome is characterized by autoantibody-dependent defects in haemostasis, leading to arterial or venous thrombosis. The list of factors, involved in the coagulation process and possible clearance of dying cells, is growing. Among several receptor molecules, which were shown to mediate uptake of apoptotic cells, was also CD68 receptor, which is a receptor for oxidized low density lipoproteins, not yet characterized phosphatidylserine receptor and phosphatidylserine by itself. Activated platelets, which selectively expose phosphatidylserine, are efficiently opsonised by naturally occurring antiphospholipid antibodies. Anti-β2-glycoprotein antibodies are one of active opsonins for apoptotic cells, which increase the efficiency of the internalization of apoptotic cell corpses, and preferentially direct them to MHC class II loading compartments. On the other hand, annexin 5 has been demonstrated to interfere with the recognition and clearance of dying cells, increasing their immunogenicity in vivo.

Sjoegren's syndrome is a lymphoproliferative disease characterized by a destructive mononuclear cell infiltration in salivary and lachrymal glands. In animal models of MRL mice, homozygous for lymphoproliferative mutation (lpr/lpr), salivary gland lymphoid infiltration similar to human Sjoegren's syndrome has been found. In experimental as well as in human Sjoegren's syndrome high levels of CD95/CD95L expression were found in salivary glands, suggesting a mechanism of apoptotic cell death.

Diabetes mellitus type 1 results from an organ-specific autoimmune mediated loss of insulin-secreting β cells. This chronic destructive process involves both cellular and humoral components detectable in the peripheral blood, months or even years before the onset of clinical diabetes. The studies from the last ten years suggested that the neonatal wave of ß-cell apoptosis might provide autoantigens necessary for triggering β-cell directed autoimmunity. In animal models increased TNFα mediated apoptosis has been found, which has been explained by selective nitric oxide-mediated upregulation of functional CD95 molecules on β cells that are subsequently killed by CD95L-producing T cells.

Hashimoto's thyroiditis is a chronic autoimmune disease characterized by a progressive destruction of thyroid epithelial cells and reduced production of thyroid hormones. In normal thyroid an exceptionally low rate of apoptosis is found, resulting in slow biological turnover of thyrocytes. CD95 is weakly or not at all expressed in normal thyrocytes. During inflammation interleukin-1β and interferon-α induce massive CD95 upregulation. Simultaneously expressed CD95 receptor and its ligand cause apoptotic cell death by paracrine or autocrine mechanisms, while infiltrating T cells do not seem to play an executive role.

Ulcerative colitis is a chronic inflammatory gut disease with mucosal alterations and the loss of colonic epithelium. The presence of apoptotic corps in the crypt epithelium suggests an involvement of apoptosis. The expressions of CD95 on epithelial cells in ulcerative colitis and normal enterocytes are comparable. It has been proposed that possible increased sensitivity to CD95 provokes apoptosis: epithelial cell death is mediated by CD95L-expressing lamina propria lymphocytes.

Multiple sclerosis is a progressively demielinating disease of the central nervous system. Destruction of oligodendrocytes has not been completely clarified, but upregulation of CD95/CD95L pathway seems to play a major role, most likely by the initiation of apoptosis at the site of inflammation.

Some examples exist with decreased apoptosis and autoimmunity. In humans decreased CD95 apoptosis has been found in Canale Smith syndrome, including nonmalignant lymphoproliferation with autoimmunity. A defective clearance due to mutations in CD95 proteins results in prolonged survival of potentially autoreactive lymphocytes. High levels of soluble CD95, found in rheumatoid arthritis patients, contribute to inhibition of synoviocyte and inflammatory cell apoptosis. An inadequate apoptosis due to defective CD95 may promote extended survival of synoviocytes. Additionally, their responsiveness to CD95L is decreased by TGFβ, interleukin-1β and TNF-α. Both lead to growth of hyperplastic pannus and destruction of cartilage.

4.8 Conclusion

Apoptosis or programmed cell death represents a natural way to eliminate unwanted cells. The elimination does not occur without any vestige and reactions. Even more, evidence has been accumulated showing that apoptotic death is involved in (auto)immune response, leading to systemic and organ specific autoimmune diseases. We are still far from the complete understanding and interpretation of all signals and processes in the course of apoptosis and their influences on autoimmunity. Faults at different stages, including defficiencies in the release or in response to endogenous molecules excreted by uncleared dying cells seem to be involved.

Literature

1. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell. 4th ed., Garland Science, 2002.
2. Kalden JR, Herrmann M. Apoptosis and autoimmunity. From mechanisms to treatments. Wiley-VCH, Weinheim 2003.
3. Horvitz HR. Worms, life and death. Nobel Lecture, Dec 8, 2002.
4. Zakeri Z, Lockshin RA, Benitez-Bribiesca L (eds). Mechanisms of cell death. The New York Academy of Sciences, New York 1999; 887.
5. Grodzitsky T, Elkon KB. Apoptosis: A case where too much or too little can lead to autoimmunity. The Mount Sinai Journal of Medicine 2002; 69:208-19.
6. Rovere-Querini Dumitriu IE. Corpse disposal after apoptosis. Apoptosis 2003; 8:469-79.
7. Hayashi T, Faustman DL. Implications of altered apoptosis in diabetes mellitus and autoimmune disease. Apoptosis 2001; 6:31-45.

5. CELIAC DISEASE - GENERAL AND LABORATORY ASPECTS

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5.1 Introduction

Celiac disease is a chronic immune-mediated disorder in genetically susceptible individuals induced by gluten proteins present in wheat, barley, and rye. Celiac disease is associated with HLA DQ2 and DQ8. It is a common lifelong disorder characterised by a variety of clinical presentations, among which are gastrointestinal symptoms and anemia. The prevalence of the disease (symptomatic and asymptomatic) is estimated to be close to 1%. Anti-transglutaminase IgA (or anti-endomysial IgA) in combination with total serum IgA is the preferred screening test for identifying individuals who need to undergo endoscopic duodenal biopsy examination., which is needed for definitive diagnosis. The pathologic lesion is characterised by a flattened small intestinal mucosa with a lymphocytic infiltrate, crypt hyperplasia, and villous atrophy. Untreated celiac disease is associated with increased morbidity and mortality (due to gastrointestinal tract malignancies). The pathologic changes and symptoms resolve when gluten is excluded from the diet.

5.2 Gluten

Celiac disease is triggered by ingestion of wheat gluten or related proteins present in rye and barley. Gluten is the water insoluble seed storage protein in wheat. Gliadin is the alcohol soluble component of gluten, and is the most toxic component of gluten. Gluten and related proteins in rye and barley are responsible for the favourable cooking and baking properties of these grains.

5.3 Clinical presentation

The clinical manifestation of celiac disease is variable and may involve multiple organ systems. The disease may present at any age. Delays in diagnosis are common.

The classic presentation associated with celiac disease is characterised by steatorrhea, abdominal distention, edema, and extreme lethargy. Nowadays, diarrhoea occurs in less than 50% of patients at presentation. Also weight loss is now a less common feature than in the past and is associated with extensive disease. In some patients, overweight is seen at the time of diagnosis. Abdominal pain, bloating, distention, anorexia, constipation, and altered bowel habit may occur in the absence of malabsorption.

In the atypical form of celiac disease, which is now common, gastrointestinal symptoms may be less pronounced or absent and extraintestinal features are more prominent. It is important that the atypical features of celiac disease are recognised.

A distinctive example of an extraintestinal manifestation is dermatitis herpetiformis. This disease is characterised by a pruritic rash on the elbows, knees, buttocks, and scalp and the presence of granular IgA deposits in the dermal papilae.

Iron deficiency anemia is a common finding in celiac disease and may be the only presenting sign. Iron is absorbed by the proximal small intestine, the site of the greatest damage in celiac disease. Besides, there might be a deficiency of vitamin D, vitamin B12, and folate. Other presentations are unexplained short stature and developmental delay in children, infertility and recurrent fetal loss, recurrent mouth ulcers, dental enamel defects, osteoporosis, fatigue, protein calorie malnutrition, and elevated transaminases.

Celiac disease may also be associated with autoimmune disorders (type I diabetes, autoimmune thyroid disorders, Addison disease Sjögren's syndrome, primary biliary cirrhosis, autoimmune hepatitis, autoimmune cholangitis), and cardiac disorders (autoimmune myocarditis, idiopathic dilated cardiomyopathy). Celiac disease has also been reported in patients with unexplained neurological complaints (cerebellar ataxia, neuropathy, epilepsy, migraine).

Genetically associated diseases include IgA deficiency, Down syndrome, Turner syndrome, and Williams syndrome.

Some individuals may have no symptoms at all and can be labelled as having silent or asymptomatic celiac disease. These patients have villous atrophy that may be discovered during intestinal biopsy for other reasons, or as a result of serologic screening of high-risk individuals. Individuals who are antibody (IgA anti-endomysial) positive but with normal or minimally abnormal small bowel biopsy examination, have been described as having latent or potential celiac disease.

5.4 Complications of celiac disease

All cause mortality among patients with clinically diagnosed celiac disease is about 2 times that of the control population. The increased mortality has been attributed to gastrointestinal tract malignancies, especially intestinal non-Hodgkin lymphoma. Enteropathy-associated T-cell lymphoma is rare and occurs in adult patients with celiac disease. Some evidence suggests that a gluten-free diet may reduce lymphoma risk. There is also an increased risk of adenocarcinoma of the small intestine, the pharynx, and the esophagus. The increased mortality has been associated with delayed diagnosis.

5.5 Prevalence of celiac disease

There is scarcity of data on the incidence of the full spectrum of celiac disease, including classical, atypical, silent and latent forms. Recent studies using serology and small intestinal biopsy suggest that the prevalence of celiac disease in Europe and in the United States is 0.5%-1%. This included both symptomatic and asymptomatic individuals. There is probably a substantial number of undiagnosed cases in the general population (possibly 10 times as many as actually have been diagnosed).

Certain populations have an increased prevalence: first degree relatives (4%-12%), type I diabetes mellitus (3%-8%), and Down sydrome (5%-12%). Furthermore, celiac disease is associated with i.a. IgA deficiency and autoimmune disorders.

5.6 Genetic factors

Genetics clearly play a role in the pathogenesis of celiac disease. The presence of specific alleles at the DQ locus appears to be necessary, although not sufficient, for the phenotypic expression of the disease. HLA-DQ2 or –DQ8 is present in almost all individuals with celiac disease. DQ2 is present in approximately 90%-95% of celiac disease patients and DQ8 in the remaining 5%-10% of patients. The DQ2 heterodimer that confers celiac disease susceptibility is formed by a β chain encoded by the allele DQB1*02 (either DQB1*0201 or 0202) and a α chain encoded by the allele DQA1*05. The HLA-DQ8-associated heterodimer is formed by a β chain and α chain encoded by DQB1*0302 and DQA1*03, respectively.

The presence of HLA-DQ2 or DQ8 is not helpful as a positive predictor of disease, as about 30% of the general population has HLA-DQ2 or DQ8 and only about 1:30 people with these genes have celiac disease.

5.7 Antibody markers

Anti-gliadin and endomysial antibodies are associated with celiac disease and are helpful in the diagnosis and management of the disease. Although anti-reticulin antibodies were used formerly, they have been replaced by the anti-endomysial antibody test in many laboratories. The anti-endomysial antibody test is an indirect immunofluorescence test. As substrate, human umbilical cord or monkey esophagus is used. In 1997, tissue transglutaminase was identified as the target antigen of the anti-endomysial antibodies.

Since then, ELISA systems for detection of anti-endomysial antibodies have been developed. In the first generation ELISAs, guinea pig tissue transglutaminase was used whereas the second generation ELISAs are based on the use of human recombinant antigen.

The diagnostic performance of the serologic markers for celiac disease varies depending on the study. Recently, a systematic and rigorous review of the literature, in which only studies that used biopsy as the gold standard were included, has been published (Hill ID, Gastroenterology 2005; 128: S25-S32).

Table 5.1. summarises the sensitivities and specificities reported in this study for the various antibodies for celiac disease. The IgG anti-gliadin antibody has a sensitivity and specificity of about 80%. IgA anti-gliadin antibody has a sensitivity that is comparable to the IgG anti-gliadin antibody, but the specificity is higher. Anti-endomysial IgA and anti-human recombinant tissue transglutaminase IgA are the most sensitive (> 90%) and specific (> 95%) serologic tests. Testing for IgG-anti-gliadin, IgG anti-transglutaminase, IgG-endomysial antibodies is useful for diagnosing celiac disease in IgA-deficient individuals.

Table 5.1. *Sensitivity and specificity of anti-gliadin IgG, anti-gliadin IgA, anti-endomysial IgA, and anti-tissue transglutaminase IgA for the diagnosis of celiac disease. The data are obtained from Hill ID (Gastroenterology 2005; 128: S25-S32) who reported a systematic review of articles written from 1966 to 2003. Inclusion in the systemic review required that diagnosis of celiac disease was confirmed by biopsy and that control individuals had normal histological findings on small bowel intestinal biopsy examination.*

*Assays based on guinea pig antigen and human recombinant antigen. It should be mentioned that assays based on human recombinant antigen are more sensitive and more specific than assays based on guinea pig antigen.

5.8 Diagnosis of celiac disease

It is important that clinicians are aware of and recognise the clinical spectrum of celiac disease. All diagnostic tests need to be performed while the patient is on a gluten-containing diet. The first step should be a serologic test. Anti-human recombinant tissue transglutaminase IgA or anti-endomysial IgA are the most accurate serologic tests. It should be mentioned that anti-tissue transglutaminase (and anti-endomysial) antibodies may miss a rare patient with celiac disease. It is helpful to also measure total IgA. If IgA deficiency is found, measurement of IgG anti-gliadin or IgG anti-tissue transglutaminase (or endomysial) antibodies is recommended. The performance of serology in children younger than 5 years is less well known and requires further study.

Biopsies (at least four) of the proximal small bowel (second part of duedenum or beyond) are indicated in individuals with positive serology. In an individual with suggestive symptoms and a negative serology test, an alternative serologic test should be performed and/or a small intestinal biopsy conducted. The characteristic histological findings are blunted or flat villi (villous atrophy), hyperplastic crypts, loss of surface enterocyte cell height, and a lymphocytic infiltration of the lamina propria. Some degree of villous atrophy is necessary to confirm a diagnosis of celiac disease. Although not used universally, the Marsh classification can be applied to standardise the pathology reports. A Marsh I lesion indicates a lymphocytic infiltration with normal mucosal architecture. Marsh II lesion exists when there is, in addition to a lymphocytosis, crypt hyperplasia. Marsh III lesion is characterised by villous atrophy. Marsh IV lesion is a rare finding and is associated with refractory disease and development of enteropathy-associated T-cell lymphoma.

A presumptive diagnosis of celiac disease can be made based on concordant positive serology and positive biopsy results. Definitive diagnosis is confirmed when symptoms improve with a gluten free diet. The serum antibodies generally disappear by 6 to 12 months, although they do not reliably reflect mucosal response. Many clinicians consider that demonstration of

normalized histology following a gluten-freee diet is no longer required for a definitive diagnosis of celiac disease. However, demonstration of histologic improvement can assure the diagnosis in patients who did not present with classical clinical features. Gluten challenge is not performed routinely now, unless there is a diagnostic difficulty, for example in patients who were already on a gluten free diet but in whom no initial diagnostic biopsy was performed.

Demonstration of the characteristic abnormalities on biopsy is key to the diagnosis of celiac disease. If histologic examination yields equivocal results, HLA typing can be useful. A negative result for HLA-DQ2 or HLA-DQ8 has an excellent negative predictive value for the disease.

5.9 Who should be tested for celiac disease?

Patients who present with the classical gastrointestinal symptoms of celiac disease and/or with atypical symptoms of celiac disease (see above) should be tested. Besides, symptomatic individuals in populations at higher risk for celiac disease (e.g. autoimmune endocrinopathies, first- and second degree relatives of individuals with celiac disease, Down syndrome) should also be tested for celiac disease. Because current data do not indicate a outcome benefit of early detection and treatment of asymptomatic individuals in these groups, routine screening cannot be recommended at this time. Similarly, there are insufficient data to recommend screening of the general population for celiac disease at this time. The long-term benefits of early detection of celiac disease and treatment with a gluten-free diet in asymptomatic individuals are not proven.

5.10 Pathogenic mechanism

Dietary gluten proteins in wheat and similar proteins in rye and barley are the triggers of celiac disease in individuals with the disease susceptibility HLA-DQ2 and HLA-DQ8 alleles. Based on the current knowledge, the following model can be put forward. Gluten peptides that are rich in proline and glutamine and not fully digested by gastric and pancreatic enzymes reach the small intestinal mucosa, most probably because of increased intestinal permeability, as can occur after gastrointestinal infection. The glutamine-rich gluten peptides are deamidated by tissue transglutaminase, which is released during tissue repair associated with infection. This tissue transglutaminase-driven modification is an important step in the immune response in celiac disease as the resulting deaminated and thus negatively charged peptides have a high affinity for HLA-DQ2 and HLA-DQ8 molecules (on dendritic cells, macrophages, and B cells) that are involved in presenting these peptides to CD4(+) T cells. Recognition of HLA-bound gluten peptides by CD4(+) T cells leads to their activation and release of cytokines. The cytokines (i) induce an inflammatory response (with release of matrix metalloproteinases that cause epithelial cell damage) and (ii) activate the production of antibodies by B lymphocytes. In celiac disease, CD8(+) and CD4(-)CD8(-) T cells infiltrate into the epithelium and probably play a role in lymphocyte-mediated destruction of epithelial cells and mucosal damage. The resulting tissue injury leads to further release of tissue transglutaminase.

In addition to having deaminating activity, tissue transglutaminase also has cross-linking activity. This cross-linking activity of tissue transglutaminase is involved in various functions, such as wound healing and stabilisation of the extracellular matrix (e.g. by cross-linking of

collagen molecules). Tissue transglutaminase is therefore expressed during tissue injury. Its expression is elevated in intestinal biopsy samples from patients with celiac disease. Tissue transglutaminase can form covalent complexes with gliadin, due to its cross-linking activity. The anti- tissue transglutaminase immune response might be generated by epitope spreading through intermolecular help, where gliadin acts as a carrier protein for tissue transglutaminase. The role of the antibodies to the mucosal lesions is not clear.

5.11 Treatment of celiac disease

Patients should not start a gluten-free diet until a definite diagnosis has been reached. Treatment of celiac disease is strict lifelong adherence to a gluten-free diet. Food products containing wheat, rye, or barley must be avoided. Grains that can be used for substitution include rice, corn, quinoa, and buckwheat. Adherence to a gluten-free diet is difficult as wheat flour is ubiquitous in foods. A dietician should be consulted.

Literature

1. Alaedini A, Green P. Narrative review: celiac disease: understanding a complex autoimmune disorder. Ann Intern Med 2005; 142: 289-98.
2. Anderson RP. Coeliac disease. Australian Family Physician 2005; 34: 239-42.
3. National Institutes of Health consensus development conference statement on celiac disease, June 28-30, 2004. Gastroenterology 2005; 128:S1-S9.
4. Dewar DH, Ciclitira PJ. Clinical features and diagnosis of celiac disease. Gastroenterology 2005; 128:S19-S24.
5. Fasano A. Clinical presentation of celiac disease in the pediatric population. Gastroenterology 2005; 128:S68-S73
6. Rewers M. Epidemiology of celiac disease: what are the prevalence, incidence, and progression of celiac disease? Gastroenterology 2005; 128:S47-S51.
7. Hill ID. What are the sensitivity and specificity of serologic tests for celiac disease? Do sensitivity and specificity vary in different population? Gastroenterology 2005; 128:S25-S32.
8. Kagnoff M F. Overview and pathogenesis of celiac disease. Gastroenterology 2005; 128: S10-S18.

6. LABORATORY STANDARDS IN DIAGNOSIS AND THERAPY MONITORING OF RHEUMATOID ARTHRITIS

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6.1 Introduction

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

6.2 The pathophysiology of RA

Genetic studies have pointed to the association of RA with HLA-DRB 10404 and DRB 10401 alleles of the MHC II complex. The basic function of HLA class II molecules is presentation of peptide antigens to CD4+T lymphocytes, which predominate in the synovial membrane infiltrates of RA patients, suggesting that RA is induced by an as yet unidentified arthritogenic antigen. This antigen may be of an exogenous (viral protein) or endogenous (citrullinated peptides) origin. The antigen activated CD4+ T cells stimulate monocytes, macrophages and synovial fibroblasts for the production of cytokines (IL-1, IL-6, TNFα), secretion of matrix metalloproteinases mediated by surface receptors (CD69 and CD11) and release of soluble mediators (IFNγ, IL-17). They also stimulate B lymphocytes by surface contact for immunoglobulin production, including rheumatoid factor (RF). Furthermore, activated CD4+ T cells express on their surface osteoprotegerin ligands, which stimulate osteoclastogenesis. Among the cytokines released by activated mononuclear cells, TNFα and IL-1 have been postulated to play a primary role in the pathogenesis of RA, based on their high concentrations in serum and synovial fluid of RA patients. They also are potent stimulators of mesenchymal cells such as synovial fibroblasts, osteoclasts and chondrocytes, stimulating their release of metalloproteinases that act destructively on the surrounding tissue. Furthermore, TNFα and IL-1 inhibit the production of tissue metalloproteinase inhibitors by synovial fibroblasts. By the induction of IL-11 release, TNFα may stimulate osteoclastogenesis, which then leads to bone degradation.

6.3 Articular lesions in RA

Figure 6.1. *Schematic presentation of normal knee joint (a); joint in early RA (b); joint in established RA (c). (From: Choy EHS, Panayi GS. Mechanisms of disease: cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 2001;344:907-16)*

In a normal joint (Figure 6.1.a) synovia consists of synovial membrane (1 or 2 cell layers) and a lower layer of loose connective tissue. The cells lining the synovia are known as A synoviocytes (macrophage-like synoviocytes) and B synoviocytes (fibroblast-like synoviocytes). In the early stage of RA (Figure 6.1.b), hyperplasia of the synovial membrane (a layer of 10 or more cells in thickness) occurs. The connective tissue layer underlying the membrane is exposed to massive infiltration with mononuclear cells (T and B lymphocytes, macrophages and plasma cells), which stimulate, by their cytokine release, the expression of adhesion molecules on the endothelial cells of the synovial vasculature. This, in turn, results in an increased neutrophil infiltration.

Furthermore, they activate angiogenesis, which additionally contributes to this effect. Neutrophils release elastases and proteases, which degrade proteoglycans in the surface layer of the cartilage. Proteoglycan depletion leads to immune complex precipitation in the surface layer of collagen and exposure of chondrocytes. Stimulated by TNFα and IL-1, chondrocytes and synovial fibroblasts release destructive metalloproteinases, primarily stromelizine and collagenases as well as catepsins. The formation of such a locally invasive synovial tissue, so-called pannus, is a characteristic histologic lesion in established RA (Figure 6.1.c). Initially, the cartilage penetration with synovial pannus consisting of mononuclear cells and fibroblasts occurs. This cellular pannus is presumed to mostly originate from B synoviocytes, in which the lack of contact inhibition of proliferation has been demonstrated by in vitro assays. This feature is consistent with the transformed cell phenotype. In the later stage of disease, cellular pannus is being replaced by fibrous pannus, which consists of a minimally vascularized layer of pannus cells and cartilage covered with a layer of collagen.

6.4 The diagnosis of RA

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

The diagnosis of RA is based on the revised (1987) classification criteria set by the American College of Rheumatology (ACR) (Table 6.1.).

Table 6.1. *The ACR 1987 revised criteria for the classification of rheumatoid arthritis*

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

The diagnosis is confirmed if 4 of these 7 criteria are present and persist for at least 6 weeks. Unfortunately, the ACR criteria are unsuitable for early diagnosis, which is crucial because irreversible damage of the joints with destruction of the cartilage begins at this stage of the disease. Therefore, the European League of Arthritis and Rheumatism (EULAR) recommends findings of more than 3 swollen joints, tenderness at metatarso- or metacarpophalangeal joints, and morning stiffness of more than 30 min for a well-founded suspicion of RA. Modern treatment of RA is being shifted towards aggressive antirheumatic therapy at an early stage of the disease because irreversible joint damage develops within the first two years. Because the potentially toxic second-line drugs can cause serious side effects, a highly specific diagnostic test that would identify patients at high risk of a destructive form of disease would be desirable.

6.5 Autoantibodies in the diagnosis of RA

6.5.1 Rheumatoid factor

Rheumatoid factor (RF) is an antibody to antigen determinants of the IgG Fc fragment. The most common isotypic profile in RA is the concomitant presence of IgM and IgA, or of IgM, IgG and IgA antibodies. In clinical practice, only IgM antibodies (Figure 6.2.) are generally determined by the methods based on RF reaction with Fc fraction of human or animal (rabbit) antibody. Agglutination tests such as Waaler-Rose test with rabbit IgG coated sheep erythrocytes, or latex test with human IgG coated latex particles can be qualitatively or semiquantitatively (titration) performed, whereas immunonephelometry and ELISA are used for quantitative measurements.

Figure 6.2. *Schematic presentation of IgM-rheumatoid factor*

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

The specificity, sensitivity and predictive values of RF as an RA marker suffer from some limitations. So, RF is positive in 60% to 80% of RA patients, indicating that a negative result does not rule out the diagnosis of RA. Furthermore, RF is positive in other rheumatic (Sjögren's syndrome, MCTD, systemic lupus erythematosus) and nonrheumatic diseases (mixed cryoglobulinemia type II – monoclonal IgM RF, chronic liver diseases, subacute bacterial endocarditis, other bacterial, viral and parasitic infections). RF is positive in up to 5% of healthy individuals under age 50, and in up to 25% of those older than 70 years. Thus, positive RF as an isolated finding without clinical criteria has no diagnostic relevance. However, RF determination is useful in the differential diagnosis of rheumatic diseases, and as a prognostic factor because its high titer is associated with rapid progressive articular destruction and extra-articular manifestations (subcutaneous rheumatic nodes, polyneuropathies, vasculitis, etc.). RF specificity for RA increases with positive RF finding on two consecutive determinations, increased RF titer, reactivity with rabbit and human IgG (positive Waaler-Rose and latex test results), and distribution among IgM, IgA and IgG classes. Although some studies have shown a high IgG RF titer to be a risk factor for the development of vasculitis, while IgA correlates with bone erosions, differentiation of RF isotypes does not contribute significantly to the prognostic value of RF determination.

6.5.2 Antibodies to cyclic citrullinated peptides (anti-CCP)

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

The target antigen was present in the cornified layer of the stratum corneum. The sensitivity of AKA test is 36% to 59%, and specificity 88% to 99%.

Two arguments have supported the concept that AKA and APF are directed against immunologically related antigens: (a) the antigens are localized in similar types of squamous epithelium; and (b) high correlation for the presence of AKA and APF in serum of RA patients. Indeed, it has been demonstrated that the antigen recognized by AKA and APF is filaggrin (filament-aggregating protein), a protein synthesized during terminal differentiation of mammalian epithelial cells and involved in the organization of cytoskeletal structure. Therefore, AKA and APF antibodies are more correctly referred to as "antifilaggrin" (AFA) antibodies.

Synthesized as a highly phosphorylated precursor protein named profilaggrin, deposited in granules, filaggrin is being released via proteolytic cleavage during cell differentiation. In this stage about 20% of arginine residues are deiminated to citrulline by the action of the peptidylarginine deiminase enzyme (PAD). In 1998, Schellekens et al. demonstrated citrulline to be a major constituent of the antigenic determinants recognized by AFA antibodies. There are at least 5 subtypes of PAD enzyme expressed in a cell type/tissue specific manner. Therefore, expression of this ubiquitous modifying enzyme in synovial cells as well as in hematopoietic cells infiltrating synovia during articular inflammation is not unexpected. The mechanism of the autoantigenic epitope formation probably involves the development of immune tolerance to the nonmodified protein. On cell differentiation, arginine residues are modified into citrulline and, if presented to the immune system (e.g., on massive cell damage or uncontrolled apoptosis), may induce immune response. However, filaggrin expression is restricted exclusively to squamous epithelium, and is not expected in synovial cells. Recent studies suggest that PAD enzyme most likely causes local citrullination of synovial proteins like fibrin, histones and vimentin. Besides AKA and APF, another RA specific antibody, anti-Sa antibody, was also found to be targeted to citrullinated protein, in this case vimentin, a cytoskeletal intermediate filament protein found in mesenchymal cells.

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

6.6 Biological markers for the management of RA

To assess the disease severity, a number of standardized rating systems according to pain and mobility indices are in use. Laboratory monitoring of RA patients is still limited mainly to inflammation parameters such as CRP and ESR, which are not joint specific and are poorly correlated with cartilage damage. Radiography of the hands and feet is still a gold standard for assessing joint damage in RA, although it allows neither early detection of joint tissue damage nor efficient monitoring of treatment.

Specific and sensitive biochemical markers reflecting abnormalities in the metabolism of joint cartilage could have a major role in the detection of early damage and thus in identifying patients at a high risk of progressive type of RA, who are candidates for disease-modifying anti-rheumatic drug (DMARD) therapy. Also, such markers could be useful for monitoring drug efficacy. Besides the promising role of anti-CCP, some cartilage turnover biomarkers could serve for the same purpose.

Articular cartilage is avascular, non-innervated hyalin cartilage tissue in which chondrocytes are located in lacunae of the extracellular matrix. Cartilage matrix contains water (up to 70%), collagen fibers, proteoglycans, non-collagenous matrix proteins and lipids. The principal structural component of cartilage is an extensive network of collagen molecules arranged in fibrils. A large aggregating proteoglycan, aggrecan, resides within the fibrillar network. The predominant collagen in the extracellular matrix of articular cartilage is type II collagen. Like collagen type I, it is synthesized as a pro-form, with propeptide extensions at both N-terminus (PIINP) and C-terminus (PIICP).

Measurement of circulating levels of these propeptides can be used as a marker of collagen type II synthesis. Mature collagen type II consists of a triple helical structure with short telopeptides at both ends. The telopeptides are covalently cross-linked to other collagen strands forming a rigid fibrillar network. Cleavage of type II collagen triple helix by collagenases results in the generation of neoepitopes at the cleavage sites. The final cleavage products are released from the cartilage tissue and can be detected by immunoassays in body fluids (synovial fluid/serum/urine).

Besides type II collagen, the second major component of the articular cartilage extracellular matrix is the proteoglycan aggrecan. It is composed of a protein (core protein) and glycosaminoglycan (GAG) chains that are covalently attached to the core protein. Fragments of the aggrecan molecule released to body fluids can also serve as markers of cartilage turnover. Finally, several noncollagenous proteins, including cartilage oligomeric matrix protein (COMP) and human cartilage glycoprotein-39, also called YKL-40, are synthesized by both chondrocytes and synovial cells, and therefore serve as markers of upregulated cartilage turnover. A summary of cartilage turnover biomarkers and their possible clinical utility in RA (based on published clinical studies) is presented in Tables 6.2. and 6.3.

Table 6.2. *Biomarkers of cartilage degradation*

Table 6.3. *Biomarkers of cartilage synthesis*

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

Literature

1. De Rycke L, Peene I, Hoffman IEA et al. Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations. Ann Rheum Dis 2004; 63:1587-93.
2. Kastbom A, Strandberg G, Lindroos A, Skogh T. Anti-CCP antibody test predicts the disease course during 3 years in early rheumatoid arthritis (the Swedish TIRA project). Ann Rheum Dis 2004; 63:1085-9.
3. Garnero P, Rousseau JC, Delmas PD. Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. Arthritis Rheum 2000; 43:953-68.
4. Christgau S, Garnero P, Fledelius C et al. Collagen type II C-telopeptide fragments as an index of cartilage degradation. Bone 2001; 29:209-15.
5. Johansen JS, Kirwan JR, Price PA, Sharif M. Serum YKL-40 concentrations in patients with early rheumatoid arthritis: relation to joint destruction. Scand J Rheumatol 2001; 30:297-304.
6. Wu W, Billinghurst RC, Pidoux I et al. Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. Arthritis Rheum 2002; 46:2087-94.
7. Skoumal M, Kolarz G, Klinger A. Serum levels of cartilage oligomeric matrix protein. A predicting factor and a valuable parameter for disease management in rheumatoid arthritis. Scand J Rheumatol 2003; 32:156-66.
8. Lindqvist E, Eberhardt K, Bendtzen K et al. Prognostic laboratory markers of joint damage in rheumatoid arthritis. Ann Rheum Dis 2005; 64:196-201.

7. LABORATORY STANDARD IN THE DIAGNOSIS AND THERAPY MONITORING OF AUTOIMMUNE DISEASE: VASCULITIS

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7.1 Antineutrophil cytoplasmic antibodies (ANCA)

Antineutrophil cytoplasmic antibodies (ANCA) are a heterogeneous group of circulating autoantibodies directed against proteins of cytoplasmic granules or other cytoplasmic and nuclear constituents of neutrophils. ANCA were first described in a few patients with segmental necrotizing glomerulonephritis. Later, ANCA were described in patients with primary systemic small vessel vasculitides-SVV (Table 7.1.).

Table 7.1. *Classification of primary vasculitides according to the Chapel Hill Consensus Conference*

Subsequently, ANCA was also described in a wide range of connnective tissue disease (CTD), inflammatory bowel disease (IBD), autoimmune liver diseases and infectious diseases (Table 7.2.). Accumulated data support the hypothesis that ANCA and their target antigens may be implicated in the pathogenesis of at least primary small vessel vasculitides.

Table 7.2. *Disorders that are different from the ANCA-associated small vessel vasculitides for which positive results for ANCA by IIF and/or ELISA have been described*

7.2 ANCA test methodology and their target antigens

Currently, three basic assay principles are applied for the detection of ANCA.
Indirect immunofluorescence (IIF) is the original and the most widely used method of ANCA detection. IIF tests are difficult to standardize, interpret and do not identify with certainty the specific antigen responsible for ANCA immunofluorescence patterns. Image analysis is an automated alternative to conventional IIF. The technique quantitates fluorescence in a single dilution of a patient sample in comparison with the intensity of standardized calibrators. Readings correlated well with ANCA levels as measured by IIF, direct enzyme-linked immunosorbent assay (ELISA) and capture ELISA. However, multicenter comparative studies are lacking.

Enzyme-linked immunosorbent assay (ELISA) is used for the determination of ANCA specific target antigen (s). Two types of such solid-phase assays were used. The target antigen can be coated directly onto plastic microtiter plate (standard ELISA) or it can be linked to the reaction well via target antigen-specific mouse monoclonal or rabbit polyclonal antibodies ("capture" ELISA or sandwich ELISA). Because the purification of native proteinase 3 (PR3) and myeloperoxidase (MPO) is laborious and requires large amounts of granulocytes, recombinant PR3 and MPO were used as target antigen in "capture" ELISA. However, ANCA assays based on these recombinant antigens have not been subjected to rigorous standardization procedures and, besides local applications, have not yet been routinely used. Other detection methods, such as immunoblotting (IB) or immunoprecipitation are not widely used for routine ANCA testing.

The "International consensus statement on testing and reporting antineutrophil cytoplasmic antibodies (ANCA)" advocates that all laboratories screen for ANCA by IIF on ethanol-fixed human neutrophils, and that any sera with ANCA fluorescence should be tested for both of the major ANCA specificities, PR3 and MPO, by ELISA.

According to the statement, ANCA recognized four different immunofluorescence patterns: a coarse granular cytoplasmic fluorescence with accentuation between the nuclear lobes - classical cytoplasmic or C-ANCA (Fig 7.1.A, e, f); a diffuse (flatter) cytoplasmic fluorescence without accentuation of the interlobular fluorescence or glanular staining - atypical C-ANCA (not shown); a typically perinuclear fluorescence with some nuclear extension (Fig 7.1.B, e) and granular cytoplasmic fluorescence on formalin-fixed neutrophils (Fig 7.1.B, f)- perinuclear or P-ANCA; pronounced nuclear rim fluorescence with the center of nucleus unstained (Fig 7.1.C, e) and non-reactivity with formalin-fixed neutrophils (Fig 7.1.C, f) -very perinuclear or "atypical" P-ANCA and atypical ANCA which include all other IIF reactivity, most commonly a combination of cytoplasmic and perinuclear fluorescence (not shown).

Relations between ANCA staining patterns and their most common molecular targets are summarized in Table 7.3. Classical C-ANCA staining patterns are found characteristically in sera from most patients with WG, but also to a lesser extent in the sera from other necrotizing vasculitides. The target antigen recognized by most C-ANCA positive sera has been identified as PR3, a neutral serine protease present in the azurophilic granules of neutrophils. Proteinase 3 has been cloned and was shown to be a 29 kD glycoprotein of 228 aminoacids. Human antibodies to PR3 (PR3-ANCA) appeared to recognize conformational determinants on the molecule.

Very rarely, sera with PR3-ANCA reactivity can also cause P-ANCA fluorescence pattern, and vice versa, MPO-ANCA sometimes can give rise to a similar C-ANCA staining pattern makes testing with anti-PR3 as well as anti-MPO-ELISA relevant. A C-ANCA staining pattern may also be seen when ANCA directed to bacteridical/permeability-increasing protein (BPI) are present. In the case of chronic infections (subacute bacterial endocarditis or cystic fibrosis) testing for BPI-ANCA may explain positive IIF results and alleviate concerns about primary SVV.

Figure 7.1. *Characteristic fluorescence patterns of ANCA on ethanol (e) and paraformaldehyde (f) fixed human neutrophil cytospin preparations. (A) a coarse granular fluorescence with accentuation between nuclear lobes (e and f) – C-ANCA; (B) a typically perinuclear fluorescence with some nuclear extension (e) and a granular cytoplasmic fluorescnce on formalin fixed neutrophils (f) – perinuclear P-ANCA; (C) a prononuced nuclear rim fluorescence with the center of nucleus unstained (e) and negative fluorescence on formalin fixed neutrophils (f) – «atypical» perinuclear – a/P-ANCA or very perinuclear P-ANCA.*

P-ANCA staining pattern is the result of a redistribution of cationic hydrophilic substances such as MPO, elastase and lysozyme onto the oppositely charged nucleus after permeabilization of cells by ethanol. This ANCA reactivity was found in most patients with MPA and iNCGN, some patients with CSS and a few WG patients. "Atypical" P-ANCA staining patterns with different frequency were found in patients with IBD, autoimmune liver diseases, infectious diseases and connective tissue diseases (CTD) such as SLE and RA. MPO represents the P-ANCA target antigen with the greatest clinical utility because of the frequent association of MPO-ANCA with SVV. All serum samples should be assayed in PR3-ANCA and MPO-ANCA ELISAs, since about 5% of serum samples are positive only by ELISA. However, many sera that produce P-ANCA or "atypical" P-ANCA staining pattern on ethanol-fixed neutrophils do not contain autoantibodies to MPO or PR3 as tested by antigen-specific assays. Recent studies show that a number of these sera contain autoantibodies

directed against a multiplicity of neutrophil constituents. In particular, autoantibodies to human leukocyte elastase (HLE), cathepsin G (CG), lactoferrin (LF), lysozyme (LZ), azurodicin (AZ), α-enolase, catalase, actin, tropomyosin, high motility groups of non-histone chromosomal proteins 1 and 2 (HMG1 and HMG2), bactericidal/permeability-increasing protein (BPI), lamin B1, histone H1 and 50 kD nuclear envelope membrane protein (Table 7.3.) Recently, a new term it has been proposed for this autoantibody population, namely "neutrophil-specific autoantibodies-NSA".

Table 7.3. *Antineutrophil cytoplasmic antibodies (ANCA) staining patterns and associated target antigen in patients with systemic vasculitides and nonvasculitic disorders*

WG-Wegener's granulomatosis; MPA-microscopic polyangiitis; CSS-Churg-Strauss syndrome; UC-ulcerative colitis; CD-Crohn's disease; PSC-primary sclerosing cholangitis; SLE-systemic lupus erythematosus; RA-rheumatoid arthritis; AIH-autoimmune hepatitis; HLE-human leukocyte elastase; BPI-bacteridical/permeability-increasing protein; HMG1/2-high mobility group of non-histone chromosomal proteins 1 and 2

7.3 ANCA as diagnostic markers

Diagnostic significance of both PR3-ANCA and MPO-ANCA for SVV is not questionable (Table 7.4.). The diagnostic relevance of ANCA depends on the clinical ordering guidelines for ANCA testing. The positive predictive value (PPV) of IIF ANCAs for SVV was very low (55-59%), with negative predictive value (NPV) between 84% and 99%, during ANCA testing in a routine clinical setting. Adherence to clinical ordering guidelines for ANCA testing restricted to patients with a reasonably high likelihood of SVV (Table 7.5.) was reported to reduce the number of false-positive tests by 27%, without missing any cases of SVV. The total number of tests performed may thus be reduced by more than 20%.

The most clear-cut association of a disease with ANCA directed against a specific target antigen is the association between WG and PR3-ANCA. Between 80% to 95% of all ANCA in WG is C-ANCA. The use of more sensitive PR3-ANCA methods (capture ELISA) of detection has confirmed that the C-ANCA in WG is almost always associated with anti-PR3. An estimated 5-20% of ANCA in WG may be P-ANCA, which are mostly directed against

MPO and only rarely directed against human leukocyte elastase (HLE). The sensitivity of C-ANCA/PR3-ANCA for WG is related to the extent, severity and activity of disease. In a meta analysis of C-ANCA in WG, the pooled sensitivity was 91% for the subset of patients with active disease compared to 63% for those with inactive disease. The specificity and positive predictive value of C-ANCA/PR3-ANCA for WG are very high (Table 4.). Most patients with MPA, iNCGN and CSS are ANCA positive, either with specificity for MPO or PR3. Other target antigens for ANCA in patients with SVV, such as BPI and AZ, may simultaneously occur with PR3-ANCA and MPO-ANCA. Whereas ANCA

Table 7.4. *Disease associations of C-ANCA (PR3-ANCA) and P-ANCA (MPO-ANCA) in primary systemic small vessel vasculitides*

PPV-positive predictive value

Table 7.5. *Clinical ordering guidelines for ANCA testing.*

as detected by IIF is found frequently in other inflammatory diseases, PR3-ANCA and MPO-ANCA are only rarely detected in disorders other than SVV. MPO-ANCA is also found in

patients with anti-GBM disease. Futhermore, false-positive MPO-ANCA may be found occasionally in patients with SLE or RA and in other inflammatory disorders such as autoimmune liver disease and inflammatory bowel disease. These false positive results, however, can be avoided by using a capture ELISA to detect MPO-ANCA. For PR3-ANCA, the capture ELISA system does not seen to differ much from a direct ELISA system with respect to specificity but the capture ELISA seems to be more sensitive. Even though the presence for WG, MPA and/or CSS, a positive ANCA result should always be interpreted with consideration of the clinical setting since the presence of specific clinical patterns plays a major role in determinig the diagnostic probability of vasculitis.

7.4 Prognostic value of ANCA during follow-up

ANCA-associated vasculitis has a 1-year survival of at least 80-90%. Treatment consists generally of a combination of prednisolone and cyclophosphamide, or other immunosuppressive drugs. Since all drugs that are used produce toxic side effect, medications are generally tapered and eventually eliminated in most cases. However, during folow-up, up to 80% of the patients in remission experience relapses. Patients with WG relapse more frequently than patients with MPA or renal limited vasculitis. In addition, patients with PR3-ANCA have more frequent relapses than patients with MPO-ANCA. This is also true when patients are subdivided into groups according to their diagnosis. So, patients with either WG and MPA who are PR3-ANCA positive have a higher relapse rate than patients with MPO-ANCA associated with the respective disease type. Thus, ANCA testing is not only a highly sensitive and specific test for making a diagnosis of WG, MPA or CSS, but ANCA antigen specificity has also a prognostic value with respect to the development of relapses during follow-up. Patients with PR3-ANCA may be at higher risk of death and patients with MPO-ANCA may be at higher risk for renal failures. Futhermore, relapses in PR3-ANCA positive patients are much more fulminant than relapses in MPO-ANCA positive patients. The causes of progression of renal failure differ between PR3 and MPO-ANCA positive patients. In patients with PR3-ANCA renal function is stable during remission, but declines with every relapse. In patients with MPO-ANCA, a slowly progressive course is often observed during follow-up without signs of clinically active disease. In these patients, proteinuria is the most important risk factor for renal failure during follow-up. In addition to ANCA specificity, ANCA levels at diagnosis and during follow-up have been shown to be predictive for patients renal and disease-free survival. A high PR3-ANCA level in capture ELISA at diagnosis is a risk factor for poor patient and renal survival and a constantly elevated MPO-ANCA level is a risk factor for poor renal survival. During induction therapy ANCA levels fall and become negative in many patients within the first few months. Persistent or recurring C-ANCA during the first year is significantly related to subsequent relapse. More than 80% of the patients who were ANCA positive at diagnosis and who experience a relapse, are test positive for ANCA at the time of relapse. So, the patients persistently negative for ANCA have a very low risk of develop relapse, although relapses localized to the respiratory tract can occur in these patients.

7.5 Prediction of disease activity by serial measurement of ANCA levels

Relapses have a major impact on disease outcome in patients with SVV. Renal relapses during follow-up have recently been shown to be the most important predictor of long-term renal survival. Therefore, it is extremely important to identify patients at risk of relapse. The usefulness of serially measuring ANCA titers in predicting disease activity is at present still

controversial. Several studies, most of them restrospective, have been published in which the relation between rises in ANCA levels as measured by IIF or by ELISA and disease activity of ANCA associated vasculitis was studied (Table 7.6. and 7.7.).

Table 7.6. *Relationships between increases in ANCA as determined by IIF and relapse of ANCA-associated small vessel vasculitis as reported by different studies*

Table 7.7. *Relationships between increases in ANCA as measured by ELISA and relapse of ANCA-associated small vessel vasculitis as reported by different studies*

N.R. - not reported

Some studies clearly show association between rise in ANCA titer and renewed disease activity, but in other studies this is not clear. When comparing IIF, direct ELISA and capture ELISA for the prediction of relapses, capture ELISA has been found to be somewhat more accurate in predicting relapses than in other techniques.

The best way to monitor PR3-ANCA, however, is probably when a combination of two methods is used. The pre-emptive therapy in PR3-ANCA positive patients when ANCA rose 75% or more clearly reduced the risk to relapse. However, during follow-up, when immunosuppressive therapy was tapered and/or stopped, ANCA titers rose again and relapses occurred. Altogether, the predictive value of measuring ANCA levels in an individual patient is not yet completely determined. Currently, the treatment based on ANCA levels alone is not yet recommended, but significant increases should prompt the clinician to monitor more precisely the condition of the patient.

7.6 Pathogenic potential of ANCA

Although direct evidence for the pathogenicity of ANCA in vasculitis is lacking, ample evidence from in vitro and in vivo studies support a potential role of ANCA in the development of vascular lesions.

PR3-ANCA and MPO-ANCA are able to inhibit enzymatic activity and prevent the inactivation of PR3 and MPO by its natural inhibitor alfa-1 antitrypsin and ceruloplasmin, respectively. In vitro, sera or purified IgG from ANCA-positive patients, as well as, monoclonal antibodies directed against MPO or PR3, have been found to induce an oxidative burst and degranulation in healthy human neutrophils pretreated with inflammatory cytokines such as tumor necrosis factor-α, IL-1β or bacterial lipopolysaccharide (LPS). Furthermore, ANCA-activated PMN are capable of damaging cultured endothelial cells. Indeed, freshly isolated and untreated PMNs from patients with ANCA-associated vasculitis are found to produce significantly more superoxide than PMN from normal control subjects. ANCA have been shown to activate monocytes to production of reactive oxygen species, IL-8, a potent attractant for PMN, and monocyte chemoattractant protein 1 (MCP-1), even without prior priming. Immunopathological studies have shown that inflammatory infiltrate is composed mainly of activated T lymphocytes, the majority of which are CD4+ and macrophages. T-lymphocytes isolated from WG patients proliferate in response to a crude neutrophil extract containing PR3 and MPO.

Although all of the aforementioned mechanisms may be operative in vivo in idiopathic vasculitis, conclusive evidence for the pathogenicity of ANCA awaits, however, a convicing animal model of ANCA-induced vasculitis. In a rat model of autoimmunity, the administration of mercuric chloride (HgCl2) to Brown Norway (BN) rats leads to a syndrome characterized by the presence of autoantibodies against a variety of antigens, including DNA, collagen, thyroglobulin, glomerular basement components and MPO. On pathologic examination of the animals, moderate acute tubular necrosis and lymphocytic infiltration in the interstitium and perivascularly can be observed. Autoantibodies against human MPO that cross-react with rat MPO, can be observed in BN rats immunized with human MPO. The autoimmune response alone does not result in clinical lesions. However, upon administration of human MPO and its substrate H202, these rats develop necrotizing glomerulonephritis with interstitial tubulonephritis, pulmonary and gastrointestinal vasculitis. Recently, it has been shown that Wistar-Kyoto rats immunized with purified human MPO in CFA develop alveolar lung haemorrhage and a mild glomerulonephritis. Moreover, recent studies on mouse models provided elegantly prove that MPO-ANCA alone induce pauci-immune glomerulonephritis and vasculitis. MPO deficient mice were immunized with mouse MPO and circulating anti-murine MPO antibodies were developed. Adoptive transfer, either of splenocytes or purified IgG derived from the MPO-immunized MPO-deficient mice, resulted in the development of crescentic glomerulonephritis and systemic vasculitis mimicking the human disease.

Schematic presentation of an integrative view of ANCA-mediated vascular tissue damage is shown in Figure 7.2. The model is based on four prerequisities for endothelial cell damage by ANCA: 1) the presence of ANCA; 2) expression of target antigens for ANCA on primed neutrophils and monocytes; 3) the necessity of an interaction between primed neutrophils and endothelium by means of β2-integrins and 4) activation of endothelial cells.

Figure 7.2. *Schematic presentation of an integrative view of the immune mechanisms involved in the pathogenesis of ANCA-associated vasculitis. The cytokines released due to infection or other tissue injury cause the priming of neutrophils and/or monocytes (A) and upregulation of adhesion molecules (ELAM-1, ICAM-1, VCAM-1) on the endothelium. Circulating primed neutrophils and/or monocytes express ANCA antigens (PR3, MPO), adhesion molecules (LFA-1, VLA-4) and FcγR on the cell surface (B). The binding of ANCA to primed neutrophils and/or monocytes induces the release of cytokines such as IL-8, IL-1β, MCP-1 and possibly other factors that are strong chemoattractants for more inflammatory cells possibly leading to granuloma formation (C). Adherence of primed neutrophils and/or monocytes to the endothelium followed by activation of these cells by ANCA. Activated neutrophils and monocytes release reactive oxygen species (ROS), which leads to endothelial cell injury and eventually to necrotizing inflammation (D). PR3 and MPO from ANCA-activated neutrophils and/or monocytes result in the endothelial cell activation, endothelial cell injury, or even endothelial cell apoptosis (E). PR3 and MPO serve as planted antigens resulting in in situ immune complexes, which in turn attract other neutrophils (F). The mechanism by which ANCA production is triggered and perpetuated remain unclear. However, T-cells are thought to play a significant role in mediating the production of ANCA by plasma cells, which are derived from antigen-specific B-cells (G).*

7.7 Concluding remarks

ANCA directed against PR3 and MPO can be detected in patients with WG, MPA including renal limited vasculitis and CSS. These ANCAs are highly specific for SVV and are the only ANCAs with clearly documented clinical relevance. Both PR3-ANCA and MPO-ANCA as tested by ELISA, but not ANCA detected only by IIF, are important diagnostic markers for these forms of vasculitis. Changes in levels of PR3-ANCA and possibly also MPO-ANCA, are related to changes in disease activity, although this correlation is far from absolute. Treatment decisions should be based on the clinical presentation of the patient and histologic findings and not exclusively on the results of ANCA testing. Immunosuppressive treatment of patients with SVV should not be guided by sequential changes in ANCA titers.

However, a rapid increase in ANCA titers or the reappearance of ANCA after a period of ANCA negativity should alert the clinician to the possibility of a relapse and thus may lead to further diagnostic procedures or shorter intervals between follow-up visits. Both in vitro and

in vivo experimental data strongly support a pathogenic role for ANCA in SVV. In vivo experimental mouse model has demonstrated that MPO-ANCA directly induces glomerulonephritis and vasculitis.

Literature

1. Boomsma MM, Damoiseaux CMGJ, Stegeman AC, Kallenberg MGC, Patnaik M, Peter BJ, Cohen Tervaert WJ. Image analysis: a novel approach for the quantification of antineutrophil cytoplasmic antibody levels in patients with Wegener`s granulomatosis. J Immunol Meth 2003; 274:27-35.
2. Cohen Tervaert WJ, Damoiseaux J. Autoimmunity-Vasculitis. In: Measuring Immunity, Basic Biology and Clinical Assessment, Lotze TM, Thomson WA (eds), Elsevier Acad Press London, 2005, 560-8.
3. Csernok E, Ahlquist D, Ullrich S and Groos LW. A critical evaluation of commercial immunoassys for antineutrophil cytoplasmic antibodies directed against proteinase 3 and myeloperoxidase in Wegener`s granulomatosis and microscopic polyangiitis. Rheumatol 2002; 41:1313-7.
4. Hagen ChE, Daha RM, Hermans J, Andrassy K, Csernok E, Gaskin G, Lesavre Ph, Lüdemann J, Rasmussen N, Sinico AR, Wiik A, van der Woude JF, for the EC/BCR Project for ANCA Assay Standardization. Kidney Int 1998; 53:743-53.
5. Hewins P, Cohen Tervaert WJ. Is Wegener`s granulomatosis an autoimmune disease? Curr Opin Rheumatol 2000; 12:3-10.
6. Hoffman SG, Specks U. Antineutrophil cytoplasmic antibodies. Arthritis Rheum 1998; 41:1521-37.
7. Huugen D, Cohen Tervaert WJ, Heeringa P. Antineutrophil cytoplasmic autoantibodies and pathophysiology: new insights from animal models. Curr Opin Rheumatol 2003; 16:4-8.
8. Malenica B, Rudolf M, Kozmar A. Antineutrophil cytoplasmic antibodies (ANCA): Diagnostic utility and potential role in the pathogenesis of vasculitis. Acta Dermatovenerol Croat 2004; 12:294-313.
9. Pollock W, Clarke K, Gallagher K, Hall J, Luckhurst E, McEvoy R, Melny J, Neil J, Nikoloutsopoulos A, Thompson T, Trevisin M, Savige J. Immunofluorescent patterns produced by antineutrophil cytoplasmic antibodies (ANCA) vary depending on neutrophil substrate and conjugate. J Clin Pathol 2002; 55:680-3.
10. Reumaux D, Duthilleul P, Roos D. Pathogenesis of diseases associated with antineutrophil cytoplasm autoantibodies. Human Immunol 2004; 65:1-12.
11. Russell AK, Specks U. Are antineutrophil cytoplasmic antibodies pathogenic? Experimental approaches to understand the antineutrophil cytoplasmic antibody phenomenon. Rheum Dis Clin North Am 2001; 27:815-32.
12. Savige J, Gillis D, Benson E, Davies D, Esnault V, Falk JR, Hagen Ch, Jayne D, Jennette ChJ, Paspaliaris B, Pollock W, Pusey Ch, Savage SOC, Silvestrini R, van der Woude F, Wieslander J and Wiik A for the International Group for Consensus Statement on testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA). Am J Clin Pathol 1999; 111:507-13.
13. Schmitt HW, van der Woude JF. Clinical applications of antineutrophil cytoplasmic antibody testing. Curr Opin Rheumatol 2003; 16:9-17.
14. Stegeman AC. Anti-neutrophil cytoplasmic antibody (ANCA) levels directed against proteinase-3 and myeloperoxidase are helpful in predicting disease relapse in ANCA-associated small-vessel vasculitis. Nephrol Dial Transplant 2002; 17: 2077-80.
15. Schönermarck U, Lamprecht P, Csernok E, Gross LW. Prevalence and spectrum of rheumatic diseases associated with proteinase 3-antineutrophil cytoplasmic antibodies (ANCA) and myeloperoxidase-ANCA. Rheumatol 2001; 40:178-84.

16. Wiik A. Neutrophil-specific autoantibodies in chronic inflammatory bowel diseases. Autoimmun Rev 2002; 1:67-72.

8. MULTIPLE SCLEROSIS

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8.1 Diagnosis of multiple sclerosis

Multiple sclerosis (MS) is the most common acquired autoimmune demyelinating disease of the central nervous system (CNS) characterized by slowly progression. The cause of MS is not known but is believed to involve 3 factors: genetic vulnerability (inheriting too many susceptibility genes and too few protection genes), some form of exposure to 1 or more environmental pathogen triggers, and the development of pathologic host immune responses directed against the CNS.

MS is heterogeneous disease demonstrating clinical variability, based on distinct clinical subtypes and disease severity. Besides subliclinical (asymptomatic) MS, that is based on autopsy studies and may account for up to 20% of MS, the most common clinical (symptomatic) form is relapsing remittiting type of disease, seen in 85% of MS at the onset, and in 55% of overall MS. This type of MS is characterized by disease attacks and clinical stability in between. Much rare is primary progressive subtype and it is seen in 10% of MS. This type of disease is characterized by slow worsening from the onset and distinctive disease onset features; older age onset, progressive myelopathy, equal gender ratio and no relapses. Five percentages of patients suffer progressive relapsing subtype which is indistinguishable from primary progressive except for superimposed relapses. Secondary progressive subtype develops in prior relapsing patient who transitioned to slow worsening. It is seen in 30% of all MS and ultimately in 90% of untreated relapsing MS.

The most important for the diagnosis of MS are clinical criteria. Important are signs of dissemination of the clinical symptoms in the space and in time. Although symptoms of MS are various and not unique, some of them are highly characteristic (table 8.1., 8.2.).

Table 8.1. *Clinical characteristic of multiple sclerosis*

Table 8.2. *Clinical symptoms in multiple sclerosis*

Data from Paty D, Studney D, Redekop K, and Lublin F. Presenting symptoms in multiple sclerosis. Ann Neurol 1994; 36:S134.

Development of clinical abnormality in relapsing remitting course of the disease is, as it is believed, influenced by number of fibers subserving the particular function and the proportion which is blocked by the demyelination or in other words clinical symptoms appears when the involvement of fibers is more than their "safety margin". When demyelination do not exceed safety margin of the nerve conduction, patients is asymptomatic.

The correct diagnosis of MS is very important because today it is well known that the best prognosis have patients with the early start of treatment with disease modifying drugs such as interferon beta (IFN) 1 a, 1 b, and glatiramer acetate (Copaxon).

To increase the specifity of diagnosis and to minimize the number of false diagnose, besides clinical, paraclinical criteria are used, the latter involving information obtained from magnetic resonance imaging (MRI), evoked potentials, and cerebrospinal fluid analysis.

Patient with MS have typical white matter lesions on MRI in over 90 percent of cases.

Conventional MRI techniques detect them as T2 hyperintense lesions (which have little to no pathologic specificity), T1 hypointense lesions (which, when chronic, indicate greater tissue damage and axon loss), and gadolinium contrast-enhancing lesions (indicating a focal major breach of the blood brain barrier and current disease activity) (Fig. 8.1.).

Figure 8.1. *MRI in MS*

However, unconventional techniques (DTI diffusion tensor imaging, MR spectroscopy) can detect microscopic and physiologic changes in normal-appearing CNS tissue. In brain MRIs of patients with MS, up to 70% of normal-appearing white matter may actually be abnormal. Cerebral or spinal demyelination, so called plaque demonstrated on MRI, when active, contains perivascular infiltrates of lymphocytes (predominantly T cells) and macrophages, occasionally also plasma cell, with prominent perivascular and interstitial edema. Most lesions seen in MRI correlate with pathologic lesions.

Figure 8.2. *Extensive demyelination of the brain tissue*

It is not entirely clear what the T2 "lesions" of MS represent. They really mean a "rearrangement" of hydrogen ions, i.e. water, and may result from the fact that myelin is hydrophobic, and when it is lost the neuropil becomes hydrophilic (Figure 8.2.). This may explain why in some cases very large demyelinations in the brain remain the same during several years and without symptoms

MRI is very sensitive and specific for predicting evolution to clinically definitive MS, more than other parameters, but doesn't necessarily correlate with the degree of clinical disability. In such cases MRI may show disseminated demyelinating lesions, but without adequate clinical symptoms.

However, it is difficult to explain why quite severe lesions sometimes remain without clinical symptoms.

Although, criteria for the diagnosis of multiple sclerosis, contains rigorous magnetic resonance imaging requirements, these criteria are short in terms of guidelines as to how the cerebrospinal fluid (CSF) analysis should be performed. There are many different techniques to evaluate CSF and no consistent standard is used among the laboratories in either the testing or reporting of CSF results. All aspects of CSF analysis will help distinguish between other causes of systemic inflammation that spill over into the central nervous system and may mimic MS such as vasculitis or chronic infection.

Besides white blood count (WBC) and differentiation, protein concentration and glucose level help to round out the CSF picture together with the more specific albumin and immunoglobulin (IgG) measurements. As immunoglobulins are regarded as very important paraclinical sign in diagnosis of MS it is important to stress out that according to the recent international consensus, IgG index or any other quantitive IgG analysis is not equivalent to qualitative analysis using isoelectric focusing with immunofixation, as opposed to the previous recommendation that simply equated the IgG index with isoelectric focusing. Isoelectric focusing on agarose gel followed by immunoblotting should be the "gold standard" for detecting the presence of oligoclonal bands. Oligoclonal bands (OCB) are found in 85 to 95 % of patients with MS. They represent limited classes of antibodies against unknown antigen, and are unique in individual patient. The presence of OCBs in monosymptomatic patients predicts a significantly higher rate of progression to MS than absence of bands. It is important to know that to 8% of CSF samples from non MS patient also contains OCBs. They maybe a result of chronic CNS infections, viral syndromes, and neuropathies. The presence of OCB is not equivalent to a diagnosis of MS. There is also a lot of false positive OCB in CSF that occur because of variability in technique and interpretation of CSF findings in different laboratories.

As the most patients with MS (80%) initially present with a clinically isolated syndrome, (CIS) the symptom highly suggestive on MS, a lot of efforts has been focused in finding the parameters that may predict early conversion in MS. It was found that the presence of serum antibodies against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) in these patients may be such parameters. The presence of MOG and MBP antibodies in patients with CIS predict the early conversion in MS, and the absence of these antibodies suggests that the patient will remain disease –free for several years.

The third important paraclinical test in the diagnosis of MS are evoked potentials. Evoked potentials (Eps) are CNS electric events generated by peripheral stimulation of a sensory

organ. They are used to detect abnormal CNS function that may be clinically undetectable. The most useful and according to the recent McDonald's criteria the only valuable EP is visual evoked response (VEP). In MS about 85 percent of patient has abnormal VEP.

It is particularly useful in patients who lack clear clinical evidence of dysfunction in supraspinal level, such as those with a progressive myelopathy. Before attributing abnormal VEP to demyelination in the optic pathways, ocular or retinal disorders must be excluded.

Diagnosis of multiple sclerosis is usually easily diagnosed by using mentioned criteria but the differential diagnosis should be carried out in clinically and /or MRI and CSF atypical findings, and especially in primary progressive course of disease. Incorrect diagnosis of MS causes great psychological changes to the patient and may lead to unnecessary treatment.

CNS lesions due to other disorders (eg.ischemia, systemic lupus erythematosus, Behcet disease, other vasculitides, HTLV-I, sarcoidosis) appear similar to MS lesions on MRI. This is particularly true for ischemic lesions which make MRI criteria much less reliable for the diagnosis of MS in patients over age of 50.

Besides CNS vasculitis, where repetitive thromboembolic events may be mistaken for a relapse-remitting course of MS (Fig. 8.3.), differential diagnosis is especially difficult in patients with optic neuritis and or myelopathy.

Figure 8.3. *MRI in CNS vasculitis - SLE*

In 50% of patients in CSF elevation of protein with mononuclear pleocytosis is found. In blood tests erythrocytes sedimentation rate (ESR) is elevated.

Lyme disease (50/100 000) is characterized with relapsing course and clinical symptoms that may mimic symptoms seen in MS. MRI findings show demyelinations in CNS (Fig. 8.4.). In CSF increased protein is found in 50% of cases and elevated WBC in 80% of patients. CSF antibodies to Boreli Burgdorferi are present in 45 % of patients. Syphilis may also cause a false positive test on B. Burgdorferi.

Figure 8.4. *MRI in Lyme disease*

Neurosarcoidosis may show clinical symptoms similar to MS. MRI may be characterized by multiple demyelinating lesions in the brain mimicking MS (Fig. 8.5.). In blood tests elevated angiotensin converting enzyme is double as normal in 60% of patients, with 5% false positive rate. In CSF OCB are positive but 80% of patients have elevated angiotensin converting enzyme levels in the CSF.

Figure 8.5. *MRI in Neurosarcoidosis*

Figure 8.6. *MRI in DEM*

Differential diagnosis is most complex at the onset of postinfectious and postvaccinal disseminated encephalomyelitis (DEM).

It may be extremely difficult to determine if the first symptoms are those of the first bout of MS or a single episode of DEM, but the nature of some symptoms and/or paraclinical findings may help. In MRI very large areas of increased signal intensity, often lobar in extent are very characteristic for DEM (Fig. 8.6.). Oligoclonal bands in CSF are often absent or if present they may disappear and that never happened in MS.

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Literature

1. Poser CM, Brinar VV. Diagnostic criteria for multiple sclerosis. Clin Neurol And Neurosurg 2001; 103:1-11.
2. Paty D, Studney D, Redekop K, Lublin F. Presenting symptoms in multiple sclerosis. Ann Neurol 1994; 36:S134.
3. McDonald I, Compston A, Edan G et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel of the Diagnosis of Multiple sclerosis. Ann Neurol 2001;50:121-7.
4. Trbojević-Čepe M. Detection of IgG bands: clinical significance and trends in methodological improvements In New trends in classification, monitoring and management of neurological diseases. The 3rd FESCC Continuous postgraduate course in clinical chemistry Handbook Ed .E. Topić 2003: 73-90.
5. Freedman M, Thompson E, Deisenhammer F et al. Recommended standard of cerebrospinal fluid analysis. Arch Neural 2005; 62.
6. Berger T, Rubner P, Shautzer F et al. Antimyelin antibodies as a predictor of clinical definitive multiple sclerosis after first demyelinating event. N Engle J Med 2003; 349:135-45.

9. LABORATORY STANDARDS IN THE DIAGNOSIS AND THERAPY MONITORING OF SYSTEMIC LUPUS ERYTHEMATOSUS

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9.1 Introduction

Systemic lupus erythematosus is the classic type of polysystemic autoimmune diseases. Lupus arises when the immune system mistakenly produces antibodies that attack the body's own tissues, including the kidneys, skin and brain. The causes of the attack are complex, and the impairment of all the main cell types of immunoregulation, T, B, dendritic cells and phagocytes are involved in this process.

The current criteria of the diagnosis are consisting of both clinical and laboratory parameters, as follows:

- Malarrash (a rash, often butterfly-shaped, over the cheeks)
- Discoid rash (a type involving red raised patches)
- Photosensitivity (reaction to sunlight in which a skin rash arises or worsens)
- Nose or mouth ulcers, typically painless
- Nonerosive arthritis (which does not involve damage to the bones around the joints) in two or more joints
- Inflammation of the lining in the lung or heart (also known as pleuritis or pericarditis)
- Kidney disorder marked by high levels in the urine of protein or of abnormal substances derived from red or white blood cells or kidney tubule cells
- Neurological disorder marked by seizures or psychosis not explained by drugs or metabolic disturbances (such as an electrolyte imbalance)
- Blood disorder characterized by abnormally low concentrations of red or white blood cells or platelets (specifically, hemolytic anemia, leukopenia, lymphopenia or thrombocytopenia) and not caused by medications
- Positive test for antinuclear antibodies not explained by drugs known to trigger their appearance
- Positive test for antibodies against double-stranded DNA, Sm or certain phospholipids or a false positive result on a syphilis test.

9.2 The pathogenesis of SLE

SLE is a multifactorial disease induced by environmental factors in patients with certain type of genetic and hormonal backgrounds.

HLA-A1, HLA-B8, HLA-DR3 genotype and C4A*Q0 complotype are the main factors of genetic predisposition for SLE.

It is a fundamental clinical observation that in the induction of SLE the role of one or more provoking factors can be recognized in every case, for example, sunlight, infections (mostly viral infections like Epstein Barr virus), hormonal, mainly oestrogen effects (the gender dependent occurrence of disease: female/male = 9/1), in addition some drugs (hydralazine and procainamid).

Though a great variety of the defects in the various signal transduction and apoptosis pathways has been described in the immunocompetents cells of SLE patients, the main problem is the loss of tolerance toward the autoantigens (the impairment of immunoregulatory T cells) and the abundant production of pathologic autoantibodies leading to tissue and organ destructions all over the body. These antibodies are of IgG type with high affinity to the autoantigens, in contrast to the "physiological" autoantibodies of IgM character and with low affinity to autoantigens and being harmless toward the tissues.

From pathological point of view all autoimmune diseases - included SLE- are special types of inflammations induced by the direct tissue damage elicited by autoreactive cells or antibodies, or by the release of various mediator substances from the white cells accumulated on the damaged area. Therefore, the results of some common laboratory tests can already give information to orient toward the diagnosis of SLE.

9.3 The common laboratory tests of inflammation used in the diagnosis of SLE

Table 9.1. *Changes in the results of common laboratory tests*

9.4 Measurement of circulating cytokines in patients with SLE

The increased activity of $CD3^+$ $CD4^+$ (ic. IL-2, IL-12, IFNγ positive) Th1 "helper" cells results in elevated serum levels of IL-1β, IL-2, IL-12, TNFα and IFN α and IFNγ. On the other hand, IL-4, IL-6, IL-8, IL-10 and IL-13 are the products of $CD3^+$ $CD4^+$ (ic. IL-4, IL-8, IL-13 positive) Th2 "helper" cells. Whereas SLE is a disease with Th1 dominance, the measurement of IL-2 and INFγ may have a diagnostic importance. Cytokine changes measured in a patient with acute SLE treated with a high dose of glucocorticosteroid are presented in the following observation gained in our laboratory:

Table 9.2. *Changes in serum levels of circulating cytokines in a patient with acute SLE after glycocorticosteroid treatment*

9.5 Changes in the peripheral distribution of lymphocytes in SLE

Flow cytometry gives the chance to determine the various subsets of peripheral lymphocytes. In addition to the above mentioned two subsets of T "helper" cells (Th1 and Th2), it is worth measuring the $CD8^+$ "cytotoxic" , $CD19^+$ "antibody producing" B and $CD56^+$ "natural killer" cells. $CD3^+HLA\text{-}DR^+$ lymphocytes represent the "late" type, whereas the $CD3^+CD69^+$ cells the "early" types of "activated" T cells. The three forms of regulatory T lymphocytes (T_{reg} cells) are the following:

a) $CD3^+CD4^+$(ic. TGFβ positive) Th3 cells,
b) $CD3^+CD4^+$ (ic. IL-10 positive) Tr1 cells,
c) $CD3^+CD25^+$ (Foxp3 positive) suppressor T cells.

Table 9.3. *Changes in lymphocyte subsets*

9.6 The types and occurrence of pathological autoantibodies in SLE

The serological verification of the various "marker" autoantibodies of SLE is the most important task of the laboratory for the diagnosis of SLE. This is a fundamental part of work proving the "autoimmune" background of the disease, furthermore, giving the chance to monitor the efficacy of therapy and to demonstrate the potential coexistence of an other autoimmune disease in a form of "overlap syndrome". We use to say that for the laboratory diagnosis of SLE it is crucial to verify one of the "marker" antibodies in the patient at least once.

The "antinuclear autoantibodies (ANA)" and "antiphospholipid autoantibodies" are forming the majority of "marker" antibodies of SLE.

Antinuclear autoantibodies (ANA or "antinuclear factor" ANF)

Antigens: double stranded (ds) DNA,
extractable nuclear antigens, Sm (U1 RNP)
nucleosome (chromatine): mixture of DNA + histone (H2A, H2B, H3, H4) molecules
nucleoplasma and nucleomatrix antigens : SS-A, SS-B.

Laboratory detection:
IF, ELISA, immunoblotting.

Antiphospholipid autoantibodies

Antigens: cardiolipin
β_2 –glycoprotein I
lupus anticoagulans

Laboratory detection:
ELISA: RIA for cardiolipin and β_2 glycoprotein I (IgG and IgM antibodies)
APTI measurement for the detection of lupus anticoagulans (prolongation).

9.7 Occurrence (per cent) of the most important autoantibodies in the sera of SLE patients

1.	**Anti-double stranded DNA (anti-dsDNA)*	60-70 %
2.	Anti-histone (H1, H2A, H2B, H3, H4)	40-60
3.	**Anti- nucleosome (anti- chromatine)*	85-90
4.	**Anti-Sm*	30-35
6.	Anti-U1RNP	20-25
7.	Anti-SS-A	40-50
8.	Anti-SS-B	20-25
9.	PCNA	1-5
10.	Anti-ribosomal P protein	12-16

11.	Rf	1-5
12.	**Antiphospholipid (anti-cardiolipin and anti-β2 glycoprotein I)*	40-50
13.	ANCA	
14.	Anti-erythrocytes	
15.	Anti-thrombocytes	
16.	Anti-endothelium	
17.	Anti-C1q	

**Marker antibodies of the disease*

9.8 Additional useful laboratory measurements for the diagnosis of SLE

Serological tests:

a. Complement measurements (C3, C4, CH50 – decrease)
b. C3a and C5a (increase)
b. Acut phase proteins (CRP – increases)
c. Circulating immunocomplexes (increase)
d. DNase activity (decreases)

9.9 Interpretation of pathological laboratory data in SLE

a) Pathological laboratory results supporting the diagnosis:

Serological tests:
Anti-ds – DNA
Anti- Sm
Anti-nucleosome (anti-chromatine)
Anti- phospholipid IgG (mainly anti-β2 glycoporotein I)
Decrease in C3, C4, CH50
Increase in C3a and C5a
Increase in CRP level and We (erythorocyte sedimentation)

Cellular tests:
Leukopenia
Lymphopenia
Thrombocytopenia
Decrease in the number of $CD4^+CD25^+$ suppressor T cells
Decreased activity and production of superoxide anions by phagocytes
LE cell positivity (atypic granulocytes phagocytosing apoptotic nucleic fragments)

Urinary tests:
Proteinuria
Cylindruria

b) Pathologic laboratory results reflecting the activity of SLE

Serological tests:

High level of anti-ds-DNA
Hypocomlementaemia
Increase in the level of C5a
Increase in the level of circulating immunocomplexes
Increased erythrocyte sedimentation

Cellular tests:

High degree of leuko/lymphopenia
Great decrease in the number of $CD4^+CD25^+$ suppressor T cells

c) Laboratory results reflecting successful therapy of SLE

Serological tests:

Decrease in the level of anti-ds-DNA
Increase in the level of C3, C4 and in the value of CH50
Decrease in the level of C5a
Decrease in the level of circulating immunocomplexes
Decrease in erythrocyte sedimentation

Cellular tests:

Increase in the number of leukocytes/lymphocytes
Increase in the number of $CD4^+$ $CD25^+$ suppressor T cells

d) Antibodies reflecting subtypes of SLE or associations with other autoimmune diseases (overlaps)

anti-SS-A: association with Sjögren syndrome (or cardiac block in neonates)
anti-histone: drug induced SLE
anti-phospholipid: antiphospholipid syndrome
p-ANCA/ anti-C1q: lupus nephritis
anti-ribosome P protein: SLE with psychiatric syndromes
cryoglobulin: SLE with vasculitis

9.10 Autoantibodies in “healthy” subjects

The specificity and sensitivity of laboratory tests applied for autoantibody determinations is strongly depend on the dilution of sera used for the measurements. It is recommended that all antibody determinations (especially the immunofluorescence measurements) should be concurrently carried out in the serum dilutions of 1:40 and 1:160. It also has to be mentioned that after infections some antimicrobial antibodies can crossreact with the autoantigens of nuclear type used in the *in vitro* assays of autoantibody determinations. These are positive reactions from laboratory point of view, but totally irrelevant from the clinical aspect of autoimmune diseases. Therefore, the close contact between the clinicians and laboratory specialists is very important in the common interpretation of clinical and laboratorial data.

9.11 The current principles of successful therapy of SLE

- Diagnosis in the earliest time (optimal co-operation between clinics and laboratory).
- Early starting of the most effective immunosuppression in order to prevent the occurrence of irreversible damages in the various organs.

List of abbreviations

ANA = antinuclear antibodies; ANCA = antineutrophil cytoplasmic antigen; ANF = antinuclear factor = ANA; APTI = activated partial thromboplastin time; CD = cluster defined; CRP = C reactive protein; DNA = deoxyribonucleic acid; DNase = deoxyribonuclease; ELISA = enzyme linked immunusorbent assay; HLA = human leukocyte antigen; ic = intracytoplasmic; IF = indirect immunofluorescence; IFNγ = interferon γ; IL = interleukin; PCNA = proliferating cell nuclear antigen; RIA = radioimmunoassay; Rf = rheumatoid factor; SLE = systemic lupus erythematosus; TGFβ = transforming growth factor β

Literature

1. Zouali M. Timing lupus. Scientific American 2005:71-7.
2. Kammer GM, Perl A, Richardson BC, Tsokos GC. Abnormal T cell signal transduction in systemic lupus erythematosus. Arthr. Rheum 2002; 46:1139-54.
3. Hoffman RW. T cells in the pathogenesis of systemic lupus erythematosus. Clinical Immunology 2004; 113: 4-13.
4. Kaplan MJ. Apoptosis in systemic lupus erythematosus. Clinical Immunology 2004; 112:210-8.
5. Bíró T, Gríger Z, Kiss E, Papp H, Aleksza M, Kovács M, Zeher E, Bodolay E, Csépány T, Szűcs K, Gergely P, Kovács L, Szegedi G, Sipka S. Abnormal cell-specific expressions of certain protein kinase C isoenzymes in peripheral mononuclear cells of patients with systemic lupus erythematosus: Effect of corticosteroid application. Scand J Immunol 2004; 60:421-8.
6. Illei G, Tackey E, Lapteva L et al. Biomarkers in systemic lupus erythematosus. Arthr Rheum 2004;50: 2048-65.
7. Manolios N and Schreiber L. Systemic lupus erythematosus. In Clinical Immunology Eds Bradly J and McCluskey J. Oxford University Press, Oxford, New York, Melbourne 1997 :329-45.

10. THE QUALITY ASSURANCE AND ORGANIZATION OF AUTOANTIBODY LABORATORY

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10.1 Introduction

There has been an enormous effort engineered in the past 20 years to find an easy, cost-effective and as-fast–as-possible way to detect autoantibodies (autoAb) especially against intracellular antigens. For years laboratory professionals have been seeking the best screen at the shortest time possible in the most cost-effective way. But, unfortunately, nobody has yet found the best solution to meet all the needs of laboratory personnel and expectations of medical specialists and insurance companies.

Autoantibody laboratory (AAL) usually services specific medical speciality:

- Systemic autoimmune
- Haematological
- Gastrointestinal
- Renal
- Liver
- Endocrine
- Cardiovascular
- Neurological
- Skin
-

Or more specific diseases:

- Antiphospholipid Syndrome
- Anti-GBM disease
- Anti-Synthetase Syndrome
- Churg-Strauss Syndrome
- Dermatomyositis
- Drug induced lupus
- Inflammatory bowel disease
- Microscopic polyangiitis
- Mixed connective tissue disease
- Polymyositis
- Primary biliary cirrhosis
- Rumatoid arthritis
- Scleroderma (diffuse, limited)
- Scleroderma, limited
- Scleroderma /Myositis overlap
- Sjögren Syndrome (primary, secondary)
- Subacute cutanous lupus erythematosus
- Systemic lupus erythematosus
- Vasculitis, systemic
- Wegener granulomatosis

When sending samples to an AAL autoAbs testing medical specialists should always be aware of the following:

- Reliability and overall quality assurance of AAL
- Repertoire of autoAb tests and methods used
- Reproducibility, comparability of test results in time (continuous care of rectification/adjustment of any changes in kits/reagents with relevant internal standards, method(s)' adaptation/ modification)
- Skilfulness in reporting test results

AutoAb tests should not be performed at primary level laboratories at Public Health departments or doctor's private practises due to:

- Complexity of organisation and management of an AAL
- Quality assurance
- Possibility of long-effective consequences of laboratory findings

10.2 Setting-up/organizing an autoantibody laboratory

In order to start and/or organize an AAL several very important premises and prerequisites should be considered:

10.2.1 Competence

Defining a doctrine of an AAL - a set of guidelines for the dynamics of autoAb testing: screening tests, specific tests for single autoAb: hierarchy of necessary tests to follow each other, what to report to a clinician. In order to implement these basic premises following questions should be answered first

- ***Where:*** hospital/institution (institutes, universities, university hospitals), independent
- ***How much:*** number of tests/year influenced by a number of medical specialists and/or hospital departments sending samples for autoAb testing. An estimation of approximate number of people potentially needing its services would be very useful.
- ***What***: routine, research, both
- ***Who:*** number of laboratory professionals: immuno-chemist (biochemist, biologist, etc), specially trained lab technicians
- ***How:*** techniques, methodology: in-house, kits
- ***To whom:*** rheumatologists, nephrologists, neurologists, other internal medicine specialists

10.2.2 Quality control

All aspects (internal, external): traceability to metrological etalons (where possible) or internationally acceptable control materials and interlaboratory comparability (national or international level, national quality assurance schemes)

10.2.3 Education

It is prerequisite to employ adequately educated (type, degree, previous training on the field) personnel with the assurance of continuous education and close relations with medical doctors in order to exchange knowledge on both parts, suggestions, information in order to make necessary changes and/or adaptations. Unfortunately, autoAb tests are not always carried out by specially trained technicians. Replacing in-house methods with kits and introducing more manufacturer and thus more kits on the market even stimulates and encourages employing cheap and thus incompetent personnel.

10.2.4 "PR (public/professional relations)"

Collaboration with similar/complementary institutions/hospitals, single/group of medical specialist(s), patient(s), other related professionals, laypersons etc.

10.2.5 Financing

Insurance companies, founds (private, institutional, governmental), research grants

10.2.6 Logistics/functioning/information system

AAL may be an advanced expert laboratory, which excellency is closely interrelated with good infrastructure and expertise of specialized medical support or, it may be estranged and melted into some large laboratory service apart from medical departments and clinicians (as an integrated part of Clinical and Biochemical Lab) and thus cut from continuous flow of knowledge and information on both sides. Adequate laboratory software for all levels of AAL functioning/activity is essential to assure an uninterrupted functioning of AAL (promptness with optimal quality, reliability, accurateness etc). To avoid any confusion defined rules should be set for the selection of big and small AAL equipment, reagents/kits, and suppliers. A transparency of all in- and out-coming people and materials (patients' sera and test results, reagents, chemicals, kits, other laboratory material, trash, dangerous waste) is obligatory and must be tracked down to the very source/beginning. Together with good software it contributes to minimizing the costs.

All the above can be more easily accomplished and facilitated by locating AAL in close vicinity of the most important customer/medical department (i.e. a division of internal medicine or even single departments, like rheumatology). It results in a very prosperous and successful collaboration in every aspect of a routine and/or research engagement. The Dept. of Rheumatology, Univ. Med. Center, Ljubljana, Slovenia with its Immunology laboratory (IL), is a good example of such successful constellation. Nevertheless, this is a very rare situation and therefore needs to be further elucidated. Autoantibody tests are much more commonly performed in a routine clinical chemistry laboratory. Most of the times, laboratory professionals (immunochemists, or immunobiochemists, or immunobiologists …) and clinicians sit on two benches separated by a wide river without seeing/understanding each other – many times there is no established collaboration between them or at least an articulation of a need to do something in this direction. A single telephone call now and than does not count: a verbal exchange of information should always take place in the form of a consultancy.

10.3 Functioning of AAL

Good laboratory practice and quality work very much depend on same issues as any other type of medical laboratory (adequately educated and well trained personnel, etc according to ISO15189). So far we have enquired about effectiveness (good services), however, functioning of an AAL crucially depends on its financing which makes it essential to take account of the efficiency of the AAL, i.e. the performance related to the expenditure. Regardless of the source of financing, ordering autoAb tests is always restrictive in approval of new and continuation of already, good established tests, due to huge and constant raise in medical costs at all levels:

Governmental/institutional laboratories: Each country/state has (not) established its own way of setting-up and organizing such type of laboratories due to current/legal health care strategy which does or does not influence insurance companies and their policy regarding payments for autoAb tests (i.e.: completely different situations in USA, EU, Eastern Europe, undeveloped world. Or inside EU: England, Germany, France, Denmark, Slovenia etc).

Private (almost always smaller) laboratories usually run very selective, single routine tests avoiding to follow guidelines proposed by the professional experts on autoAb. They tend and actually do reduce their costs on almost everything: kits are selectively approved (many times depending on "more results for less money"), constantly cutting down the costs for materials and human resources. In many countries they often hire inadequately educated and thus cheaper labour to run the tests, which often result in a very poor laboratory performance regularly neglecting quality controls on all levels. Results are unreliable, inaccurate, irreproducible, incomparable in time and inadequately interpreted. They are unable to make any quality discussion with relevant medical specialists, often ignoring and disregarding suggestions for possible further steps in quality assurance of laboratory diagnosis. It goes beyond a simple AAL/clinician communication, which usually ends up in none at all.

From all the above autoAb tests should always be performed by laboratory specialists with a history of extensive training on the field.

10.4 AAL in Rheumatology

Focusing on rheumatology as one of the main users and beneficiaries of an AAL the following autoAb tests against organ non-specific antigens should definitely be included:

a. Screening tests:
   - ANA test* (antinuclear antibodies: meaning a differentiation of imunofluorescence (IF) patterns of nuclear membrane, nucleoplasm, nucleoli, spindle apparatus and cytoplasm on Hep-2 cells together with semiquantitative evaluation of a titer).
   - Anti-ENA (antibodies against extractable nuclear antigens) **
   - ANCA* (antibodies against neutrophyl cytoplasmic antigens)

b. Tests on autoAb against specific antigens:
   - ds-DNA (double stranded DNA)
   - Sm, U1RNP, Ro, La, Scl-70, Jo-1**
   - CL (cardiolipin),
   - beta2GPI (beta2glycoprotein I),
   - LA (lupus anticoagulant)
   - CCP (cyclic citrulinated peptide)
   - MPO (myeloperoxydase)
   - PR3 (protein 3)

* Certain specific fluorescences should be confirmed by tests on specific antigens: AMA like – anti-PDH (pyruvatedehydrogenase), ribosomal like – anti-ribRNP, PCNA like – anti-PCNA (proliferating cell nuclear antigen), different ASMA IF etc; pANCA – MPO, cANCA – PR3)
** Not a screen when tested on single antigens

We believe it is of vital importance that the repertoire of an AAL is consisted of those autoAb tests that cover most diagnostic needs (i.e. rheumatology), a complete service in one place in order to assure minimal quality standards for AAL functioning from employing skilled personnel to internal and external quality controls. Namely, an adequate testing for autoAb

should always begin with "ANA" testing on Hep-2 cells which represents the first screening test, although not an ideal and not a complete one. Further testing depends on several facts and assumptions: i.e. positivity/negativity of a tested serum, (presumed) diagnosis, availability of specific test(s) in the same AAL where ANA was performed, money. Besides ANA, some specific autoAb tests should be included, based on patients' diagnosis thus forming some typical sets of initial autoAb tests. Based on national guidelines or agreement among AAL, rheumatologists and insurance companies, groups of tests can be preset in advance.

a. In an ANA positive serum there are two possibilities for further testing:

- Second screen for anti-ENA with CIE,
- Testing on single selected autoAb depending on (presumed) diagnosis (with the awareness of relevant diagnostic criteria and incidence for particular autoAb)

b. There has been no consensus how to proceed testing (if at all) of a serum declared as ANA negative. However, it is not a clear-cut decision whether a serum is positive or negative; it depends on several things:

- Method of choice (in-house, kit), manufacturer of Hep-2 cells, conjugates
- Starting serum dilution (cut-off point)
- Method accurateness
- Microscopy performances
- Skilfulness of the person reading the IF and differentiating IF patterns

Evaluation of ANA by IF begins with a starting serum dilution, which represents the cut-off. It ranges from 1:40 to 1:160 and is one of the main disagreements and differences among AALs. There are several reasons for this discrepancy and money is the most important one: selecting 1:80 or even 1:160 as the cut-off means more negatives and less further testing for autoAbs. By our opinion, declaring a serum negative at the dilution 1:40 is not the same as is at 1:80 or 1:160. For more than 20 years IL has been using 1:40 dilution as the cut-off dilution for ANA testing on Hep-2 cells (the same manufacturer of Hep-2 cells preparations). Over 17 years CIE has been suggested as the second screen for more specific anti-ENA thus giving clinicians much more information to work with, since there are no CIE false positives.

We believe that using both tests speeds up the diagnosis of those patients who would stay (at least for a long time) undiagnosed especially in cases of poor (or none at all) collaboration between AAL and clinicians (Table 10.1.). Again, it should not be solely AAL to decide about further autoAbs testing but all three parties involved: AAL, rheumatologists, financier (eventually it all ends up in higher costs).

Table 10.1. *IFANA and CIE/anti-ENA in 5431 patients in 1998/1999*

At this point it should not be overlooked the effort of a group of experts who in order to improve the quality of autoAb testing, constituted the European Consensus Study Group for Autoantibodies (Consensus group) who meet annually at the European Workshop for Rheumatology Research (EWRR). The group was formed in 1988 to examine the sensitivity and reproducibility of different methodologies for the detection of autoAb to intracellular antigens such as immunofluorescence, gel techniques (immunodiffusion (ID) and counterimmunoelectrophoresis (CIE), ELISA, Western blotting, and then to improve the performance of these tests. Therefore, every year 10 different sera samples are sent to 35—40 leading laboratories from 21 different European countries to test for as many autoAb against intracellular antigens as possible, and the results of these exercises show a tremendous improvement in detection rates. A significant step in the life of the EWRR has been reached when laboratory experts in the consensus group agreed to submit their protocols, of which the best were chosen and published in the Manual of Biological Markers of Disease. Participating laboratories often function as national expert laboratories to disseminate the knowledge from the consensus meetings to other laboratories and clinicians. One of the main conclusions of the Consensus group was that different detection techniques could not be directly compared. Immunofluorescence as a good screening method should precede all the more specific ones. Borderline results obtained by ELISA or immunoblotting tests should be further confirmed by one of the gel techniques (ID or CIE).

IL has been a part of the consensus group since the very beginning, contributing to the CIE protocol for the detection of autoAb against different intra-cellular antigens (anti-ENA). In the last 15 years, over 205.000 sera have been routinely tested for different autoAb; among them 100.000 have been screened for more than 10 different autoAb specificities by our unchanged version of CIE (5). Almost exclusively in-house methods have been used for autoAb with most satisfactory results at the lowest possible cost. The same version of the CIE method has been used in IL as the second screening test for different anti-ENAs since 1987, therefore we like to expose certain aspects of the method:

Since immune complexes formed in CIE gel precipitate as distinct lines, all the reactions between autoAb and relevant antigens are actually seen. This includes also those with unknown specificities. Therefore, many rare, but sometimes diagnostically important autoAb (PCNA, SL, Ku, PM/Scl, etc.) were discovered by simple routine testing. Considering the results, CIE is neither expensive nor complicated: it can be performed at very low cost in a very reasonable time, 24 hours for the overall positive/negative result and additional 24 hours to test for specific autoAb. This is, for this type of analytes, more than acceptable. A trained technician can test over 100 sera in just 2 hours needed for screening for positivity with two antigen substrates and to further characterisation of all positives using relevant standard antisera. The negative CIE test means that the tested serum is negative for all autoAb against most common antigens (Ro, La, Sm, U1RNP, Jo-l, Sd-70) and also against some rare, but very important ones (Table 10.2.) PCNA, PM/Scl, Mi-2, PL-4, PL-7, PL-12, Ku, SL), as well as against many unknown antigens. Therefore, there is no need to run separate tests for each autoantibody separately as is the case with ELISA technique.

Table 10.2. *Autoantibodies against some rare antigens (4)*

SLE-systemic lupus erythematosus, SS-Sjoegren's syndrome, SSc-Systemic clerosis, PM-polymyositis, DM-dermatomyositis (* An on-going multicentre study leading by Dept. of Rheumatology, Ljubljana: anti-ENA/CIE on over 600 SSc patients)

Based on over 17 years of experience, we have summarised major practical advantages and disadvantages of the two-step CIE procedure and its relationship to ELISA.

10.4.1 Advantages of CIE

1. Positive/negative testing for all known and unknown autoAb precipitating in CIE gel can be performed in a single CIE run.
2. CIE methodology can easily be adapted to run many sera in a single run (from one to 50 or more).
3. During the first CIE step defining anti-ENA positivity/negativity of samples no standard antisera are required.
4. The sensitivity of the CIE for routine purposes is excellent (10-20 µg/mL of antigen).
5. There are no false positive results: if the test is positive, it is positive for one or more of autoAb against more than 15 known and many unknown antigens.
6. CIE is easy to perform: an average technician needs only 2—3-weeks training in a specialist laboratory.
7. Since CIE is always an "in-house technique, all the test procedures (including antigen extraction) are transparent and can be readily controlled and adapted for specific purposes.
8. An individual laboratory can establish its own pool of secondary standards according to primary CDC or WHO standards.
9. CIE detects autoAb mainly against native antigens
10. By introducing different substrates and some minor modifications of the CIE procedure, we can determine antibodies against other important antigens (i.e. antibodies against pyruvate dehydrogenase complex).

10.4.2 Disadvantages of CIE

1. Some rare autoAbs, i.e. ribRNP, do not precipitate in agarose CIE gel.
2. Recombinant antigens ("single epitope" antigens) are too small to precipitate in CIE gel; therefore autoAb against such antigens cannot be detected by CIE.

10.4.3 CIE versus ELISA

The point, which is many times ignored, is that ELISA can never replace CIE: each of them has their role in autoantibody testing. Results obtained by CIE and ELISA methods cannot be compared directly: they are useful for different purposes and give different answers:

1. With CIE, all major autoAb can be detected in two CIE runs.
2. ELISA tests apply only to selected antigens, giving one answer at a time, while CIE uses cell extracts (multiple antigen substrates), each time giving multiple answers.
3. Classic ELISA, or its automated versions, can never be good screening techniques as results obtained with regard to five or more specific antigens do not mean that a particular serum is also negative for other autoAb not included into same kind of repertoire.
4. ELISA gives too many false positive results sometimes leading to inadequate medical treatment. Setting-up cut-off values is even more problematic than in IF ANA testing.
5. Some ELISA kits give unreliable results due to unacceptable variations in batch-to-batch analysis.
6. It is not the analytical sensitivity but the analytical specificity, which remains one of the major problems with different ELISAs.

Many laboratories throughout Europe introduce only ELISA tests for autoantibody detection into their routine practice, which, we think, is inadequate and may be dangerous. We evaluated anti-ENA by CIE in sera from over 5.000 patients previously tested by IF ANA on Hep-2 cells. In patients with negative and low positive ANA (titre $\leq$1:80) there was about 6% of those with positive anti-ENA on anti-Ro, Jo-1 and UDA (Table 2.).

Considering recommendations of the consensus group, each laboratory analysing autoAb to intracellular antigens should be able to perform several basic techniques:

- Immunofluorescence test on Hep-2 cells as the first screening.
- If routine tests for specific autoAb are performed by ELISAs and a particular result is problematic, it should be confirmed/retested by a second technique such as immunoblotting, or by one of the gel techniques (RID, CIE).
- The results in such cases should always be interpreted for clinicians and not just reported.

We also believe that autoantibody testing should be performed with an utmost care. Therefore, it should be in the hands of highly professional personnel in expert laboratories covering all the necessary techniques for autoantibody testing.

10.5 Conclusions

Due to the dramatic shortage of resources in health system on one side and the fast extension of laboratory tests on the other, services of an AAL is continuously pressurised to increase its efficiency. To meet the needs of all patients and clinical personnel responsible for human healthcare, minimal requirements should be set up. ISO 15189 provides particular requirements for the quality and competence of medical laboratories. For its implementation into AAL adequately educated and trained laboratory professionals is obligatory.

10.6 Take-home messages

In order to organize an AAL the following premises should be considered: competence (where, how much, what, who, to whom, how), quality control, education, financing, and logistics/informational system.

1. AutoAb tests should always be performed by laboratory specialists with a history of extensive training on the field, who is able to evaluate and interpret analytical results into laboratory (interpreted) findings.
2. The first screening test for autoAb in rheumatology is “ANA“ testing on Hep-2 cells (by indirect immunofuorescence).
3. All sera (ANA positive or negative) should be screened for anti-ENA with CIE in those patients where connective tissue disease is suspected or tested on single autoAb(s), being aware of all limitations of single autoAb testing.
4. Counterimmunoelectrophoresis is fast, easy and cost-effective method for the detection of autoAb to intracellular antigens and dramatically lowers the overall costs for autoAb search.
5. If routine tests for specific autoAb are performed by ELISAs and a particular result is problematic, it should be retested by a second technique such as immunoblotting, or by one of the gel techniques (immunodiffusion, CIE).
6. AutoAb test should be performed in a laboratory, covering all the necessary techniques for autoantibody testing - autoAb tests should not be performed at primary level laboratories at Public Health departments or doctor’s private practises
7. ISO 15189 provides particular requirements for the quality and competence of medical laboratories. For its implementation into AAL adequately educated and trained laboratory professionals is obligatory

Literature

1. International Organisation for Standardisation. Medical Laboratories – Particular requirements for quality and competence (ISO 15189: 2004)
2. Büttner J. Good laboratory practice: The medical aspects. Editorial. Eur J clin Chem Clin Biochem 1997; 35:251-6.
3. Jansen RTP, Blaton V, Burnett D, Huisman W, Queralto JM, Zerah S, Allman B. Essential criteria for quality systems in medical laboratories. Eur J Clin Chem Clin Biochem 1997; 35:121-2.
4. Wilkinson I. Managing Change in the Clinical Laboratory. IFCC series. IFCC 2005.

5. van Venrooij WJ, Charles P, Maini RN. The consensus workshops for the detection of autoantibodies to intracellular antigens in rheumatic diseases. J Immunol Meth 1991; 140:181-9.
6. Charles PJ, van Venrooij WJ, Majni RN. Consensus finding group for autoantibodies. The consensus workshops for the detection of autoantibodies to intracellular antigens in rheumatic diseases: 1989—1992. Clin Exp Rheum 1992; 10:507-11.
7. Humbel RL. Detection of nuclear antibodies by immunofluorescence. In: Van Venrooij WJ, Maini RN, editors. Manual of biological markers of disease. Dordrecht: Kiuwer Academic Publishers, 1993.A2/1-16.
8. Bunn C, KvederT. Counterimmunoelectrophoresis and immunodiffusion for the detection of antibodies to soluble cellular antigens. In: Van Venrooij WJ, Maini RN, editors. Manual ot biological markers of disease. Dordrecht: Kiuwer Academic Publishers, 1993.A3/1-12.
9. Kveder T, Božič B. Counterimmunoelectrophoresis: fast, easy and cost-effective method for the detection of autoantibodies to intracellular antigens. Clin Chem Lab Med 2002; 40:428-9.
10. Charles PJ, Maini RN. Enzyme-linked immunosorbent assay in the rheumatological laboratory. In: Van Venrooij WJ, Maini RM, editores. Manual of biological markere of disease. Dordrecht: Kluwer Academic Publishers, 1993:A5/1-19.

11. STATISTICAL MANAGEMENT OF AUTOIMMUNE DISEASES DATA

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11.1 Handling the data – statistical management

From the statistical point of view, basic purpose of research of autoimmune diseases, as it is mainly with all others, is to collect the data to obtain information about particular disorders. Usually, data are obtained from one or more samples of patients that represent the population, the whole group of individuals of our interest. Just as an example, if the topic of investigation is expression of CD45 lymphocyte isoforms in newborns shortly after they have been delivered (Juretić *et al*., Ref.), then the sample of newborns has to be representative of the same population. We collect and analyze the data on a sample of some (randomly chosen) N individuals and use them to draw conclusions about the population. That process – making inferences – is the meaning of scientific research. After reading the paper and learning something new on lymphocytes in early newborns, we expect to have the same finding in all newborns in the world (generalization), i.e., that facts from the paper, related to individuals in that research, become general facts considering all individuals with same characteristics (and that is why those characteristics of individuals have to be briefly but clearly explained in the research paper). Of course, this is a simplified explanation of generalization and we also have to recognize that information from the sample does not fully indicate what is true in the population. There is also a sampling error that has to be considered.

If the sample is not representative, if it just enumerates a group of individuals or subjects that were examined, measured, studied and analyzed, then the process of making inferences about the population does not exist at all – there is no population for which facts from the study might be true. But so-called convenience sample might be useful just to test something before it will be utilized in the real scientific research, for example, to test if new database on autoimmune diseases is user friendly by entering the data of first ten patients that come in the office. Of course, there is no statistics to be reported about these patients, only about filling the forms.

Correct sampling is only the first step. Statistical management of data obtained from biomedical research and from clinical trials is a complex knowledge of choosing an appropriate statistical method for the data analysis and data presentation. Beside statistical analysis itself, i.e., utilizing predefined mathematical calculations with collected data to compute tests' specific values with their probability values, handling the data also implicitly includes that researcher has knowledge on sampling, data and errors types, outliers, distributions, measures of average and data spread, hypothesis theory, study design, data transformations, etc. Readers of scientific papers must also have basic knowledge on statistical data handling; otherwise, reading can not be critical, as expected.

As presented recently by Tom Lang in Croatian Medical Journal, inadequate statistical reporting in scientific literature is mostly due to authors' poor knowledge about research design and statistics, statisticians' inability to communicate statistics to authors, editors and readers, lack of involvement of statisticians at the beginning of research, and not applying statistical reporting guidelines.

11.2 Statistical errors

Statistical errors in scientific reporting are not rare, as someone might expect, and some critical reviewers of biomedical literature found that about half of the articles that used statistical methods did so incorrectly. Even big, high-impact and prestigious journals are not immune on statistical errors. Most errors concern basic statistical concepts and can be easily avoided by following guidelines. Typically, guidelines are published as regular printed handbooks (for example: Lang and Secic, Ref.), they can be prepared as educational electronic manuscripts and published on Internet, or can be published in medical journals as a part of guidelines for authors (for example: Editorial Policy of Croatian Medical Journal, available in the 1st issue of each volume, but also at *www.cmj.hr*).

One good example of electronic manuscript is "Guides to Good Statistical Practice" from the Statistical Service Centre of The University of Reading (UK), intended primarily to give help to research and support staff in development projects. The guides are available to read online (*http://www.rdg.ac.uk/ssc/publications/guides.html*) or to download for printing and reading offline from the same Internet address. They were also rewritten and published in 2004 as a book (Stern *et al.*, Ref).

Some biomedical journals introduced statistical editors to confront the problem, but still without finding the perfect solution to the problem, suggesting that some other measures are necessary, such as strict editorial policy on statistical review, monitoring of revised manuscript version and enrollment of formally trained biostatistician (Lukić & Marušić, Ref).

11.3 Are autoimmune diseases data immune on statistical errors?

Probably not, but no brief and straightforward answer was found after keywords "autoimmune disease" and "statistical error" were run through Medline search: 39 papers published between 1981 and 2005, three of them, review articles, were found in database but none of them considering the topic of this paper (Fig. 11.1.). In most publications "statistical error" was considering new or corrected approach to the subject, analysis of statistical errors in procedures performed through the study, or comparative analysis of two or more techniques (only abstracts were analyzed).

Figure 11.1. *Medline search, using PubMed service on Internet*

To find an answer to the question, a small observational study on non-representative sample was performed. In between 15.208 articles published in numerous biomedical journals in years 2004 and 2005, listed by keyword "autoimmune disease" from Medline, only seven journals with high impact factors were considered in selection and only those that can be found in a printed version at the Central Medical Library of the Zagreb University School of Medicine: American Journal of Hematology, Arthritis Research and Therapy, Arthritis & Rheumatism, Autoimmunity, Diabetes, Journal of Autoimmune Diseases, and Lupus.

From 1.075 articles published in these seven journals, fifty papers were chosen by chance, but at least one per journal, to form simple convenience sample. Two young biomedical scientists, O.G. & T.M., attentively read articles and in 18 of them found at least one error related to the statistical analysis or statistical data presentation. In total, more than thirty errors were found that can be presented through six groups (mistakes, errors, doubts and ambiguities will be presented individually during the lecture, here only a summary of groups is given).

11.3.1 Data types, presentations and mismatch

This group of errors consists mostly of mistakes, typing errors and inadequate data presentations. Some are unintentional, but sometimes authors are unaware of possibility to present the data using texts, tables or graphs. Also, some problems concerning data types in making the difference between categorical and numerical data occur, resulting in wrong statistics and possible mistakes in conclusions. Some "historical tables" were still noticed, listing all data from the experiment, and with no consequent data analysis.

Numerical data might appear in two distinct types, discrete and continuous, and each of them has their own characteristics of presentation. Also, if data have measuring unit, it has to be presented.

11.3.2 Average and dispersion

Problem with summarizing the data appears when authors do not have knowledge on their own data (How big is the sample? Is distribution normal? How sampling was conducted?) or when authors do not understand types of summarizing techniques. Numerous errors were found, from small to important ones. After reading results of presentations, readers definitely can not infere from sample to population.

11.3.3 Problems with "Subjects and Methods"

Unclear sampling methods and appearance of questionable control subjects were detected. Information about statistics authors frequently write together with results, instead of putting statistics theory into the Methods section of the paper. Surprisingly, in some papers more methods of statistical analysis were discussed than afterwards was presented in the paper.

11.3.4 Statistical errors

This is probably the most important group of errors, covering all kinds of inappropriate statistics that might lead to completely wrong conclusions. Unfortunately, some were found, even one dealing with low correlation coefficient, close to zero but significant, that was considered important!

11.3.5 P-values

All kinds of errors in presenting probability values (no p-values, only statements what is significant and what is not with no numbers, improper decimals, etc.) and wrong explanations on significance were frequently noticed.

11.3.6 Results

Statistical explanation, if used as the only explanation while presenting results is wrong, indicating that author probably does not understand output of statistical analysis. Results should be presented in a way that everybody from the same scientific field can read, understand and have no doubts about them.

11.4 Instead of the conclusion

Although some authors reported that big scientific journals might differ from small ones in a preparation of manuscript for publishing, considering statistical management of the manuscript and statistical data reporting and presentation, this small *ad hoc* study proved that field of autoimmunity research still suffers from statistical unclearness. And that could bring up the next question – how much can we rely on the results we are reading about?

Literature

1. Dawson-Saunders B, Trapp RG. Basic and clinical biostatistics. 3rd ed. London: Prentice hall Int. Inc 2001.
2. George SL. Statistics in medical journals: a survey of current policies and proposals for editors. Med Pediatric Oncol 1985;13:109-112.
3. Glanz SA. Biostatistics: how to detect, correct and prevent errors in the medical literature. Circulation 1980;61:1-7.
4. Lukić IK, Marušić M. Appointment of statistical editor and quality of statistics in a small medical journal. Croat Med J 2001;42:500-3.
5. Juretić E, Gagro A, Vukelić V, Petrovečki M. Maternal and neonatal lymphocyte subpopulations at delivery and 3 days postpartum: increased coexpression of CD45 isoforms. Am J Reproductive Immunol 2004;52:1–7.
6. Lang T. Twenty statistical errors even YOU can find in biomedical research articles. Croat Med J 2004;45:361-370.
7. Lang T, Secic M. How to report statistics in medicine: annotated guidlines for authors, editors, and reviewers. Philadelphia: Am. College of Physicians 1997.
8. Petrie A, Sabin C. Medical statistics at a glance. London: Blackwell Science 2000.
9. Stern RD, Coe R, Allan EF, Dale IC, ed. Good Statistical Practice for Natural Resources Research. Statistical Service Center. Published May 2004. Paperback, 416 pages, ISBN 0851997228.

12. GUIDELINES FOR ANTINUCLEAR ANTIBODY TESTING

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12.1 Introduction

Testing for autoantibodies has become an important part of clinical diagnostics, estimation of prognosis and, thus, planning of follow-up and possible therapeutic approach. Finding a strongly expressed autoantibody in early disease where the full spectrum of clinical manifestations is yet not present can guide further exploration to reveal subclinical tissue or organ involvement and thus give a more precise overview of the incipient disease.

Some autoantibodies represent more characteristic predictors of a certain disease than a particular clinical manifestation or histopathological finding. This fact particularly pertains to the disease-specific autoantibodies. Nevertheless all positive autoantibody finding need to be set into a clinically meaningful context to be useful for clinical diagnostics. With the advent of many new and very sensitive technical platforms and assay formats for detecting autoantibodies clinicians and laboratory scientists need to collaborate closely to reveal the clinical usefulness before results coming from a new technology can be as certain and informative as the results derived by use of classical technologies (e.g. double immunodiffusion, counter-immuno-electrophoresis, passive haemagglutination etc.). Such thorough work on clinical utility must precede any introduction of new technologies and assays for diagnostics in a laboratory.

12.2 The use of antinuclear antibodies (ANA) in rheumatology

The most indispensable parts of clinical diagnostics relate to the clinical history, family history, and manifestations found clinically. Diagnostic aids such diagnostic imaging, histopathology/ immunopathology, simple laboratory tests to detect signs of inflammation, autoantibody testing and specialist evaluations are secondary to the clinical setting found at presentation. The use of one or a few screening tests - rationally ordered after setting a *tentative diagnosis* – can lead to low cost but high quality diagnostics. Simple screening for ANA using indirect immunofluorescence technique (IF) and a sensitive cellular substrate is an appropriate strategy in unfolding clinical and laboratory diagnostics. A positive result can lead to exploration for antibodies known to be important for that particular diagnosis and for the IF result found. Though the term ANA relates to autoantibodies direceted to nuclear antigens only, the term is very commonly used in a broader sense to describe any antibody giving rise to a positive staining pattern on a cellular substrate (i.e. including those thjat target cytoplasmic structures). In this presentation this broader definition of ANA will be used. The most popular cellular substrate used for such ANA screening today is the human epithelial cell line HEp-2 cells derived from a laryngeal carcinoma, and the preferred conjugate used for visualization of antibody binding is specific for human IgG.

12.3 ANA screening using HEp-2 cells.

ANA can roughly be divided into those that recognize antigens in five different regions of the cell: the *nuclear envelope*, *the nucleoplasm* with its organelles, *the nucleoli*, *the mitotic spindle apparatus* and *the cytoplasm* with its organelles. In the following I will thus use the term ANA for all of the antibodies that can be seen by IF testing using HEp-2 cells. Although the cell contains thousands of different proteins only very few of these have been found to have autoantigenic properties. The reason why cellular proteins are turned into autoantigens are partly unknown, but events taking place during inflammation and cell death seem to cooperate with a number of genes in causing this antigen transformation.

The precise recognition of a particular well-defined HEp-2 cell staining pattern on one hand can lead the laboratory scientist to determine the most likely autoantigens recognized and on the other hand indicate known relationships to a limited number of likely diagnostic entities. In this way a particular positive ANA screening result can guide further specific ANA testing but also be useful for unravelling a precise clinical diagnosis/ prognosis.

Some laboratory scientists have stated that the precise categorization of an IF staining pattern cannot be reached at by most laboratory technicians, but this is clearly wrong. With the use of reference images and unique terms for each pattern, precisely defined by a team of experts, can result in the development of very accurate recognition skills in most laboratory workers as proven by international multi-centre studies. Among the multitude of clearly defined IF patterns seen in a clinical immunology laboratory, the majority can be used directly by clinicians to promote diagnostic work-up if the laboratory has the ability to explain the most likely clinical associations seen with a positive screening ANA result. The majority of these patterns can not be detailed further by specific ANA testing using available routine enzyme-immuno-assay (EIA) technology since either the autoantigen is not clearly known or it is not available in a form that can be used in presently used kit formats. Among the many ANA patterns known only around 10-12 specific ANA targets can be detected in an EIA, immuno-blotting or line-immuno-assay format.

12.4 Use of ANA for diagnosis and estimation of prognosis

It is well known that some ANA are used as diagnostic criteria as part of a systemic rheumatic disease diagnosis e.g. *systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome (SjS),* but other ANA serve as an important diagnostic support for diagnosis e.g. *scleroderma (SSc), poly-/dermatomyositis (PM/DM), secondary SjS, secondary anti-phospholipid antibody syndrome (APAS), and juvenile chronic arthritis (JCA) (Table 12.1.).*

Table 12.1. *ANA as diagnostic criteria or support for diagnosis in rheumatic disease.*

*See text about the various ANA.

It is assumed that the ANA found in a patient with any of these diseases somehow reflect genetic predisposition and lesional pathology in a particular individual. Since involvement and severity of different organs is directly related to disease prognosis the revelation of a particular ANA in a patient can guide the clinician in the follow-up and surveillance of incipient organ manifestations so that rational therapy can be instituted early.

A specially illustrating example of such relationships is SSc, where anti-centromere antibodies mostly are associated with a slowly developing form of *limited SSc* which has a good long-term prognosis, while anti-topoisomerase I (anti-Scl-70) antibodies relate to a more rapidly progressing form of *diffuse SSc* commonly complicated by *fibrosing alveolitis* and a more cumbersome prognosis. Anti-RNA polymerase I antibodies have been found to be associated with a particularly severe form of rapidly progressing *diffuse SSc*, that commonly involves the kidneys and manifests with malignant hypertension, cardiovascular disease and cerebral infarctions. In SSc patients that harbour anti-U1RNP antibodies the disease is practically always *overlapping* with another immuno-inflammatory rheumatic disease e.g. SLE, PM/DM or MCTD, and the prognosis may be very different from case to case. Similar clinical subsyndromes have been found in SLE, primary SjS, PM/DM, JCA. Each subsyndrome is thus associated with presence of a particular specific ANA, and the nature of that ANA should be revealed if at all possible.

Some ANA can be difficult to reveal with certainty, probably due to different properties or different epitope specificities seen in relationship to a njumber of diseases. Nevertheless, credible results are absolutely necessary in order to allow a meaningful use of the serologic information. A typical example is that of anti-double stranded DNA (-dsDNA), where independent studies have reached the same conclusion, i.e. anti-dsDNA that are characteristic for SLE can only be disclosed by using at least two different methods for their demonstration.

This may be explained by the fact that production of some types of anti-dsDNA can be a normal response to certain infections or to any type of long-standing tissue injury. In our laboratory we have reached at a strategy where we start screening for anti-dsDNA using an EIA that is known to be broadly reacting and thus has a low specificity for SLE, but then a positive result is followed up by use of a *Crithidia luciliae* IF test which is highly specific for SLE if found positive. Only a if a positive IF test is found we report anti-dsDNA to be present. A positive result of the EIA oly is not reported as positive. With that approach we have shown that the sensitivity in SLE patients is around 45-50 % and the diagnostic specificity around 97 %.

12.5 How can we judge the clinical utility of results from solid phase assays?

It is clear from many reports that a positive test for ANA using EIA or some other solid phase based technology does not correspond well to what is found by the classical double immuno-diffusion or counter-immuno-electrophoresis techniques which rely on presence of precipitating antibodies. Before one can use results from such solid phase assays in clinical work-up it is necessary to explore the diagnostic potential by studying sera from local populations of immuno-inflammatory diseases. Healthy donor controls cannot be used for a clinically meaningful cut-off setting. Results derived from the study of a prototype disease must be compared with those of inflammatory disease controls that manifest features somewhat similar to the prototype disease (“critical controls”). By constructing receiver-operation curves and choosing a preferred high level of specificity cut-off can be set.

After that the sensitivity for the prototype disease can be seen from the curve. By setting a similar high level of specificity for diagnosis different assays for the same antibody can be rationally compared. Tests that are used to establish diagnosis need to have a high diagnostic specificity whereas the sensitivity is less important. Even rarely found ANA relate to a particular subsyndrome and prognosis.

It is very important to prove the value of a test for early diagnostics. In early disease a positive ANA result has a relatively higher impact on clinical decision-making than later in follow-up. The most informative ANA results are those that are unique to one diagnostic entity (disease-specific).

12.6 What should be done to establish serologic positivity in borderline cases?

Since the early start of the European consensus studies the recommendation has been to confirm or refute doubtful borderline results ("grey area" results) by e.g. performing two different techniques. Alternatively one can agree with clinicians to report such results with a written "caveat notice" that the result cannot be used with confidence for differential diagnostics. Another possibility is to agree on calling all such results "negative". This has to be discussed between laboratory personnel and clinicians, so the policy is always the same.

12.7 Use of algorithms

Collaboration between clinicians and laboratory scientists may also lead to agreement on the use of practical algorithms for test ordering, for rational stepwise exploration of a preliminary result at screening, and for interpretation of a positive final result. As an alternative to an algorithm for test ordering it may be practical to set up the order form in such a way that the doctor can tick either a tentative diagnosis or one or more single tests (Fig 12.1.). Thereby the flexibility of test ordering is maximal and people who may be uncertain about which tests will be rational to do can learn from the form.

Figure 12.1. *Choice of test packages or single specified orders on test order form.*

12.8 Use of international serum standards in the laboratory

The IUIS/WHO/CDC/AF International Committee on Standardization of Autoantibodies in Rheumatic and Related Diseases have established a repository of well-characterized freeze-dried sera ampouled into glass vials as standards or alignment tools for producing national and local serum standards. These standards can be ordered free of charge from Center for Disease Control in Atlanta, GA. Until now 14 different standards are available and in the coming year more standards will be made available.

12.9 Efforts to harmonize clinical/laboratory collaborative work

For five years annual meetings have been organized in the Nordic countries to make clinicians aware of the importance of collaborating with clinical immunology laboratories to optimize diagnostics and make the diagnostic process more appropriate for differential diagnostic use.

Clinicians and laboratory scientists have discussed a number of items that are handled very differently in different centres with the aim to harmonize such activities to the benefit of the end user, the patient. This led to the formation of a European steering group of leading scientists in rheumatology, called EASI (European Autoimmunity Standardization Initiative). Senior people from rheumatology and clinical immunology are now being recruited from hopefully every European country with the task to plan national discussions of the items laid forward as suggestions from the steering committee, hoping that controversies between different country policies can be bridged and the suggested measures amended in such a way that all nations get a unified concept of how to interact across each country. The final plan is to have European open meetings where these plans and strategies are presented by all national delegates for fruitful discussions. The 5th International Autoimmunity Congress in Sorrento next year will set up such a general session for interested parties.

12.10 Modern technical platforms and new assays.

Many new assay platforms and new technologies to detect and quantify specific ANA have been introduced by the industry, now is the time to find out what should be their role in future autoimmune serodiagnostics using the strategy outlined above. There is no doubt that precision and speed of testing can be made much better with automation, but that is just a small part of rationalizing laboratory work and may not contribute to better diagnostics. We need to know the clinical implications of getting positive results that are not substantiated by IF methodology or precipitation techniques. We need to have many more autoantigens ideally expressed on solid phases (addressable laser bead assays, multiplex assays etc.) so that true pathological ANA are binding but polyreactive low affinity (diagnostically unimportant) ANA are not. We also need to look at the possible value of quantitating various ANA as part of disease surveillance, an area of research that has been much neglected until now. Hopefully we can also start to look at pathobiological effects of certain ANA (e.g. the complement-fixing properties of lupus-related ANA) as compared to the same ANA specificity in other diseases. We know very little about the ANA found in inflamed tissues and fluids compared to the corresponding serum ANA. Until now there are no indications that high quality detection of ANA using a solid phase principle can take the place of HEp-2 cell ANA demonstration by IF, and there are multiple reasons for that.

12.11 Important issues in health cost estimation

Many scientists have wondered how to handle the increasing complexity and demand for autoimmune serodiagnostics. Many have switched from manpower-dependent to automated technical platforms trying to keep short-term costs low. One needs to realize that health costs are very low in the early phase of chronic diseases, total laboratory costs mounting to 2-3% of the patient-related costs in Sweden, whereas the heavy costs arrive during the later phases of such diseases. These long-term costs are dependent on many factors some of which are number of visits to clinics, length of stay and cost of stay in hospital, readmission rate, working days lost for the patient and family, productive years gained, economic compensation for inability to work etc. The best way to cut these long-term costs is to set an early diagnosis through the use of optimal clinical/serological diagnostics, making decisions about interventions as rational as possible and thus effect ultimate outcome.

12.12 Conclusions

ANA most likely reflect tissue lesion mechanisms, genetic predisposition and perhaps etiology, are associated to diagnosis, subsyndrome categorization, and prognosis, may help planning of clinical follow-up and therapeutic strategies, are of particular value in early diseases cases, can best be revealed by IF using HEp-2 cells, and can be credibly interpreted by non-medical personnel. Modern testing platforms are perhaps easier in use but not better. To arrive at optimal clinical diagnostics patients need to donate blood for testing purposes, and clinicians and laboratory scientists need to collaborate closely.

Literature

1. Wiik A. Anti-nuclear autoantibodies: clinical utility for diagnosis, prognosis, monitoring, and planning of treatment strategy in systemic immunoinflammatory diseases. Scand J Rheumatol 2005; 34:260-8.
2. Bizzaro N, Wiik A. Appropriateness in anti-nuclear antibody testing: from clinical practice to strategic laboratory practice. Clin Exp Rheumatol 2004; 22:349-55.
3. Wiik AS, Gordon TP, Kavanaugh AF, Lahita RG, Reeves W, vanVenrooij WJ, Wilson MR, Fritzler M, and the IUIS/WHO/AF/CDC Committee for the Standardization of Autoantibodies in Rheumatic and Related diseases. Cutting edge diagnostics in rheumatology: the role of patients, clinicians, and laboratory scientists in optimizing the use of autoimmune serology. Arthritis Rheum (Arthritis Care & Research) 2004; 51:291-8.
4. Fritzler MJ, Wiik A, Fritzler ML, Barr SG. The use and abuse of commercial kits used to detect autoantibodies. Arthritis Res Ther. 2003; 5:192-201.
5. Fritzler MJ, Wiik A. Autoantibody assays, testing and standardization. In: Roe NR, Mackay IR (eds): The autoimmune diseases, 4th edition, Elsevier, London. (in press).
6. Tan EM, Feltkamp TEW, Smolen JS, Butcher B, Dawkins R, Fritzler MJ et al. Range of antinuclear antibodies in ”healthy” individuals. Arthritis Rheum 1997; 40:1601-11.
7. Fritzler MJ, Wiik A, Tan EM, Smolen JS, McDougal JS, Chan EKL et al. A critical evaluation of enzyme immunoassay kits for detection of antinuclear antibodies of defined specificities. III. Comparative performance characteristics of academic and manufacturer’s laboratories. J Rheumatol 2003; 30:2374-81.

8. Fenger M, Wiik A, Høier.Madsen M, Lykkegaard JJ, Rozenfeld T, Hansen MS, Danneskjold-Samsoe B, Jacobsen S. Detection of antinuclear antibodies by solid phase immunoassays and immunofluorescence analysis. Clin Chem 2001;50:2141-47.

9. Pottel H, Wiik A, Locht H, Gordon T, Roberts-Thomson P, Abraham D et al. Clinical optimization and multicenter validation of antigen-specific cut-off values on the INNO-LIA™ ANA Update for detection of of autoantibodies in connective tissue disorders. Clin Exp Rheumatol 2004; 22:579-88.

10. Wiik A. Testing for ANA and ANCA – diagnostic value and pitfalls. In Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH (eds): Rheumatology, 3rd edition, Mosby, Edinburgh, pp 215-226.

11. Wiik A, Lam K. Report to the European Commission: On the usability of extended DOORS software for education and training, quality assurance and consensus formation. Deliverable D 09, version 2.1, 2001. The EU CANTOR project HC4003(HC)2000.

13. THEORY, TARGETS AND THERAPY IN RHEUMATIC DISEASES

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Autoimmunity appears to contribute significantly to many different rheumatologic diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), dermatomyositis, Sjoegren syndrome, systemic sclerosis and the antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides. The best evidence for autoimmunity is the presence of autoantibodies in a large proportion of patients. These autoantibodies are class - switched, somatically mutated, often high affinity and directed against diverse epitopes on the autoantigens - all features associated with T cell dependent immune response. There are also data, particularly in SLE, demonstrating the presence of autoantibodies, and hence autoimmunity, years before the onset of clinical disease. The present paradigm for the development of these autoimmune diseases is that autoimmunity develops in four stages: genetic predisposition, initiation, perpetuation and progression and clinical disease. B cells take up and present autoantigens via specific cell surface immunoglobulins to T cells and they can help regulate and organize inflammatory responses. The importance of these latter functions has been demonstrated in murine SLE, where B cells have been found to be critical to the development of disease even when they are unable to secrete autoantibodies.

The understanding of immunopathogenic mechanisms in autoimmune disease including SLE has increased exponentially and this has led to the discovery of novel targets for which biologic or targeted therapies have been developed against. The mainstay of therapy for severe manifestations of SLE include the use of high-dose corticosteroids and cytotoxic agents such as cyclophosphamide (CYC) which have been associated with an increased risk of serious and opportunistic infections. Since the 1980s, we have argued for more judicious use of steroids and more recently, controlled studies have demonstrated that low-dose i.v. CYC and mycophenolate mofetil are equally effective and less toxic than high dose CYC in the treatment of lupus nephritis.

The potential advantage of biologic therapy is possibly, a better safety profile with less general immunosuppression. These targeted therapies may range from small molecules that specifically inhibit inflammatory processes at an intracellular, cell-cell or cell-matrix level to monoclonal antibodies (mAb), soluble receptors or natural antagonists that interfere with cytokine function, cellular activation and inflammatory gene transcription.

The immunopathogenic hallmark of SLE is the polyclonal B cell activation which leads to hyperglobulinemia, autoantibody production and immune complex formation (Figure 13.1.). The fundamental abnormality appears to be the failure of T cells to suppress the forbidden B cell clones due to generalized T cell dysregulation with resultants excess in CD4+ T cell activity and deficient CD8+ cytotoxic/suppressor function. In addition, B and T-cell interaction is facilitated by several cytokines such as IL-10 as well as co-stimulatory molecules such as CD40/CD40L, B7/CD28/CTLA-4 which initiate the second signal. These

interactions together with impaired phagocytic clearance of immune complexes and apoptotic material perpetuate the immune response with resultant tissue injury.

Figure 13.1. *Immunopathogenesis of SLE (adapted from Moc CC et al.)*

The «prototypic» biologic agents first approved for use in rheumatic disease were the anti-tumour necrosis factor (TNF-ά) inhibitors: etanercept and infliximab, for the treatment of rheumatoid arthritis. Since the initial success, its use has been extended to the treatment of spondyloarthropathy (ankylosing spondylitis, psoriatic arthritis) and some preliminary data has emerged suggesting benefit in other rheumatic diseases such as several forms of systemic vasculitis (Behcet's disease. Churg – Strauss syndrome, and polyarteritis nodosa) and even a certain subgroup of patients with SLE. Following this lead, a new generation of biologic agents for the treatment of SLE is currently being developed, some of which have reached clinical phase trials. The following discussion on these novel therapies have been classified according to the potential targets of the immune cascade in SLE.

13.1 B cell targeted therapies

It is now clear that apart from autoantibody production, B cells play a critical role in amplifying the immune response through its function as antigen-presenting cells. Autoantigens are presented via specific cell surface immunoglobulins to T cells together with a second signal via co stimulatory molecules which leads to T cell activation. B cell blockade (Figure 1) can thus be directed at: 1) B cell surface receptors (CD-20, CD-22). 2) inhibition of

co-stimulatory signals CTLA4 Ig, 3) inhibition of B cell survival (antiBLyS). and 4) induction of B cell anergy (B cell toleragens).

13.1.1 Blockade of B-cell surface receptors

Rituximab, a monoclonal antibody against CD-20+ B cells was first approved for use in the treatment of non-Hodgkin's cell lymphoma. It selectively depletes immature, mature, naive and memory B cells. Plasma cells do not express CD-20 and are hence unaffected. There is encouraging data from open label trials and case reports demonstrating its efficacy and safety in SLE. Notably, it appears to be beneficial in those with active refractory disease and none of the studies thus far have reported significant adverse effects, particularly that of serious infection. This observation has also been supported by other recent case reports citing successful outcomes in patients with life-threatening SLE (renal, haematological and central nervous system involvement). It appears, from the studies performed, that successful depletors (patients with <1% B cells in peripheral blood) have a more sustained clinical response compared to "poor depletors" and this variable response may be related to polymorphisms of FC γ receptors as well as the dose of rituximab.

13.1.2 2. Inhibition of costimulatory signals

Cell surface molecules that mediate cell-cell interaction and generate intracellular biochemical signals in the interacting cells are termed «costimulatory molecules». These provide the much needed second signal for T cell activation by antigen-presenting cells (Figure 13.2.). The costimulatory targets that have been or are undergoing evaluation in patients with SLE include the CD40-CD30L and CD28-CTLA4-B7 molecules. CTLA4 (cytotoxic T-lymphocyte antigen4), expressed on activated T cell surfaces, provides inhibitory signals with down regulate T cell function whereas CD28-B7 interaction promotes T-cell activation. As the former has higher affinity to B7, investigators have developed CTLA4Ig, a soluble receptor (fusion protein of the extra cellular domain of CTLA4 and Fc portion of IgGI) to block CD28-B7 interaction and subsequent T cell dependent B cell function. Following promising animal date demonstrating improvement in lupus nephritis, survival and reduction in autoantibody and cytokine (IL-2, c and 10) production with CTLA41g monotherapy as well as in combination with CYC and encouraging results in patients with rheumatoid arthritis, a Phase I/II study in SLE patients conducted by NIAID is underway.

Figure 13.2. *Targets for SLE therapy (adapted from Vasoo and GRV Hughes)*

Unfortunately, the anti-CD40L mAb treatment approach in human lupus was not fruitful, as short-term administration of the anti-CD40L mAb, ruplizumab in lupus nephritis was associated with life-threatening prothrombotic activity despite initial encouraging data in the serology and renal function of the patients. Ironically, treatment with another anti-CD40L mAb (designated as IDEC-131) did not prove to be clinically effective in human, SLE, despite being well tolerated. A glimpse of other potential targets include monoclonal antibodies against CD137 costimulatory molecules on T cells in NZB/WF1 SLE-prone mice which have reversed the progression of established SLE-like disease and prolonged survival.

13.1.3 Inhibition of B cell survival

B-lymphocyte stimulator (BLyS) also known as BAFF, is a member of the TNF family of cytokines. Expression of BLyS receptors (BCMA, TACI, BAFFR) is largely restricted to B cells. It has been shown in animal models that over expression of BLyS results in a lupus-like state and knock-out mice models for BLyS ameliorates the disease. In human SLE, over expression of BLyS is common (persistent elevation in up to 50% of patients over a one year follow-up period). The results of a recent Phase I trial of fully humanized monoclonal IgG antibody against BLyS were encouraging. There was a significant decrease in peripheral B-cells and treatment was a significant decrease in peripheral B-cells and treatment was well tolerated with no serious adverse reactions. However, no clinical or serological improvement was detected in this cohort study. Currently, a Phase II clinical trial is ongoing and other BLyS antagonists are being explored for use in humans. They include BAFFR-Ig (preclinical trials) and TACI-Ig (Phase I in normal subjects).

13.1.4 Induction of B cell anergy

The introduction of synthetic molecules which have the ability to crosslink with antidsDNA antibodies on the surface of B cells leading to anergy or apoptosis is another novel therapeutic approach that is undergoing further evaluation in human SLE. LJP 394 is one such agent, composed of four deoxynucleotide sequences bound to a triethylene glycol backbone that has shown promising results in the Phase II/III clinical trials. The two large randomized, DBPC studies in patients with lupus nephritis showed that there was a significant delay in the time and incidence of renal flares, as well as reduction in anti-dsDNA Ab levels in the subgroup that demonstrated high affinity binding of anti ds-DNA to LJP 394 could be useful adjunct to current therapies for lupus nephritis but the requirement for it to be administered weekly may limit its utility. The recruitment for the Phase IV DBRCT evaluating high dose LJP 394 (100mg and 300mg) in patients with active lupus nephritis is ongoing. A similar approach is also being investigated for the treatment of antiphospholipid syndrome using LJP1082.

13.2 Complement inactivation

In SLE, complement activation follows immune complex formation and the focal point on the final common pathway is C5 with resultant formation of the membrane attack complex (MAC) C5b-9 that causes tissue damage. The blockade of C5 in SLE was first studied in murine lupus which showed significant amelioration of renal disease and marked increase in survival. A Phase I clinical trial of anti C5mAb revealed that it was safe and well tolerated with a trend to improvement in disease assessment scores in the high dose group (8mg/kg).

13.3 Cytokine modulation

Cytokines are low molecular weight mediators of cell-cell communication and include interleukins (ILs), interferons (IFNs), growth factors and others. They are heterogeneous and function in an overlapping manner. The key principle is that the net biologic response in any tissue is the result of balance between local levels of proinflammatory or anti-inflammatory cytokines. SLE is considered by some to be a Th-2 driven disease with documented elevations in serum IL-4, 6,10.

13.3.1 Anti –IL 10 mAb

Interleukin-10 (IL-10) levels are elevated in patients with active SLE and correlate with disease activity. These alterations in IL 10 and TGF beta regulation appear to result in T-cell dysfunction (accelerated T -cell apoptosis and dysregulation of T- cell dependent B- cell function). The results of a pilot study of six patients with active SLE treated with anti IL-10 mAb for three weeks and followed up for a period of six months were promising. There was marked reduction in circulating IL10 levels with corresponding clinical improvement (decline in MEX-SLEDAI and mean daily prednisolone dose) which were maximal at two months and sustained at the end of the six month review. Apart from one case of mild transfusion reaction, no serious adverse events were reported.

13.3.2 Anti IL6mAb

Interleukin-6 (IL-6) levels are elevated in both human and murine systemic lupus erythematosus (SLE). IL-6 is a potent proinflammatory cytokine that has a wide range of biological activities including terminal differentiation of B – lymphocytes into antibody - forming cells and T cells to effector cells. IL-6 blockade ameliorates disease activity in murine models of SLE. MRA is a humanized monoclonal antibody against the human IL-6 receptor. Data from clinical trials in patients with rheumatoid arthritis suggest that MRA may be an effective and relatively safe agent to block the effect of IL-6.Mild and transient transaminitis, leucopoenia and diarrhoea were observed in the treatment group. Hence, its role could possibly be extended to the treatment of SLE. An open label, dose-escalating, Phase I study of MRA in patients with moderately active SLE is currently underway to address its safety and efficacy.

13.3.3 Interferon –alfa (IFN alfa) antagonism

Recent evidence suggests that IFN-alfa may play a role in murine lupus models and human SLE. The clinical observation that some patients with malignancy or hepatitis C treated with IFN alfa developed autoimmune conditions such as SLE led to a new area of research of IFN alfa antagonism as a potential target in SLE therapy. IFN alfa has numerous biological activities. It enhanced T-cell activation, differentiation and cytokine production (IL10) which in turn activates B cells and autoAb production through a variety of mechanisms. Data from murine lupus models lends further support to this observation. IFN/ betaR knock out NZB/W F1 mice demonstrated significant improvement in serological and clinical manifestations of SLE. Theoretically, there are numerous potential levels at which IFN-alfa antagonism can be targeted but several questions need to be answered such as the possible differential regulation of INF alfa, the role of IFN-alfa subtypes in SLE, the concern of compromising anti-viral immune responses.

13.3.4 TNF alfa inhibition

The exact role of TNF alfa in the pathogenic pathway of SLE remains unclear. TNF blockade in patients with RA or Crohn s disease has led to the development of lupus-like illness, development of antinuclear and anti-ds DNA Abs (although invariably IgM, not IgG) in some patients. On the contrary, treatment of murine lupus with anti TNF therapy resulted in therapeutic benefit and the preliminary results of a recent open pilot study of six refractory SLE patients (four with nephritis, three with arthritis) were give four doses of IV Infliximab at 300mg each, showed a 60% reduction in proteinuria at the end of the follow-up period as well as remission of arthritis and disease activity, despite the expected rise in anti-dsDNA Abs. It is noteworthy however that three out of the six patients developed urinary tract infections, complicated by E.coli bacteraemia in one patient. A plausible explanation may be that a subset of SLE patients exists in which TNF over activity is pre-eminent and hence respond to TNF blockade. At present however, there are insufficient data to recommend widespread use of such agents in SLE.

13.4 Gene therapy

Preclinical studies have provided proof of concept that gene therapy in SLE is feasible and effective. Successful efforts include gene constructs that alter the expression of cytokines via i.m. injection of naked DNA encoding cytokines or adenoviral mediated gene transfer to CTLA4-Ig into murine lupus models with resultant clinical improvement. Other effort may include gene modified gene transfer such as autologous B cells transfected with toleragenic constructs or T cells in which specific molecular aberrations have been corrected.

Literature

1. Vasoo S, Huges GRV. Perspectives in the changing face of lupus mortality. Autoimmun Rev 2004; 3:415-17.
2. Bompas DT, Furie R, Manzi S et al. For the BG 9588 Lupus Nephritis Trial Group. A short course if BG9588 (anti CD40 ligand Ab) improved serologic activity and decreased hematuria in patients with proliferative lupus GN. Arthritis Rheum 2003; 48:719-27.
3. Looney RJ, Anolik JH, Cambell D et al. B cell depletion as a novel treatment for systemic lupus erythematosus. Arthritis Rheum 2004; 50:2580-9.
4. Tackey E, Lipsky PE, Illei G. Rationale for interleukin-6 blockade in systemic lupus erythematosus. Lupus 2004; 13: 339-43.
5. Schmidt KN, Ouyang W. Targeting interferon alfa in SLE therapy. Lupus 2004; 13: 348-52.
6. Aringer M, Zimmermann C, Graninger WB, Steiner G, Smolen JS. Tumor necrosis factor alfa blockade in systemic lupus erythematosus: an open-label study. Arthritis Rheum 2004; 50:3161-9.

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