Dissertation of DOCTOR OF PHILOSOPHY (Ph. D.)

# IDENTIFICATION OF GENETIC FACTORS ASSOCIATED WITH SJÖGREN'S SYNDROME

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| B-NHL | B cell non-Hodgkin lymphoma |
| bp | base pair |
| cDNA | complementary DNA |
| CTL | cytotoxic T cell |
| DMEM | Dulbecco's Minimum Essential Medium |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| G3PDH | glyceraldehyde-3 phosphate dehydrogenase |
| GM-CSF | Granulocyte-Macrophage Colony Stimulating Factor |
| HaCaT | human spontaneously immortalized keratinocyte cell line |
| HLA | Human Leukocyte Antigen |
| IFN | interferon |
| IgG | ImmunoglobulinG |
| IL | interleukin |
| MHC | Major Histocompatibility Complex |
| MnlI | restriction enzyme MnlI |
| mRNS | messenger ribonucleic acid |
| NK | natural killer cell |
| NLE | Neonatal Lupus Erythematosus |
| nt | nucleotide |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| RA | Rheumatoid Arthritis |
| RFLP | Restriction Fragment Length Polymorphism |

| | |
|---|---|
| RNA | ribonucleic acid |
| rpm | round per minute |
| RT | Room Temperature |
| SCLE | Subacute Cutaneous Lupus Erythematosus |
| SLE | Systemic Lupus Erythematosus |
| Ss | Sjögren's syndrome |
| SSCP | Single-Strand Conformational Polymorphism |
| TGF | Transforming Growth Factor |
| Th | helper T lymphocyte |
| TNF | Tumour Necrosis Factor |
| UVB | Ultraviolet B radiation |

# I. INTRODUCTION

The immune system normally can distinguish “self” from “non-self”. During the autoimmune process the immune system loses the self-tolerance ability, which may be the result of abnormal selection or regulation of self-reactive lymphocytes and by abnormalities in the way that self-antigens are presented in the immune system (Abbas-Lichtman 2003).

Sjögren’s syndrome (Ss) is a systemic autoimmune disorder that mainly affects exocrine glands and usually presents as persistent dryness of the mouth and the eyes due to functional impairment of the salivary and lachrymal glands (Daniels 92). Therefore the most important manifestations of the disease are keratoconjuctivitis sicca and oral dryness caused by diminished lachrymal and salivary gland secretory activity (Fox 99). Antibodies against ribonucleoproteins (antiRo/SSA and antiLa/SSB) are frequently present in the sera of Ss patients. In the absence of an associated systemic autoimmune disease, patients with this condition are classified as having primary Ss (Talal 93). Ss is characterized by B cell hyperactivity and hypergammaglobulinemia. Several cytokines, including IL-10, IFNγ and some autoantibodies have been proposed to have a role in the pathogenesis of the disease.

Antibodies produced against Ro/SSA and La/SSB autoantigens are not only of diagnostic value but they may even play a role in the pathogenesis of several autoimmune diseases (Sjögren’s syndrome/Ss, subacute cutaneous lupus erythematosus/SCLE, neonatal lupus erythematosus/NLE and systemic lupus erythematosus/SLE) (Martinez-Lavin 79, Sontheimer 82, Maddison 81). Among other factors, ultraviolet (UV) radiation and the hormonal milieu are well-known cofactors in the pathogenesis of these autoimmune diseases as well. Anti-Ro/SSA and anti-La/SSB autoantibodies are two of the most common antibodies found in the serum of patients with Sjögren’s syndrome (Martinez-Lavin 79). During the last

decade three genes have been isolated which encode three Ro/SSA protein antigens 46, 52 and 60 kDa (McCauliffe 93). The 46 kDa antigen is a calcium-binding protein that resides predominantly in the endoplasmic reticulum, while the 52 kDa and the 60 kDa Ro proteins are located in the cytoplasm or the nucleus. Ultraviolet B (UVB) radiation, estrogen treatment and some viral infections result in translocation of the Ro/SSA antigens to the cell surface, where they are available to bind circulating Ro/SSA autoantibodies (LeFeber 84, Furukawa 90, 88, Tesar 86). The La/SSB antigen, which is located primarily in the nucleus, is a single protein transiently associated with all RNA polymerase III transcripts, the hYRNAs (Pruijn 90). It carries autoantigenic determinants, and it has been suggested that La/SSB participates in the synthesis, maturation and nuclear export of this class of RNA molecules. In lymphocytes of patients with Sjögren's syndrome three different splicing variants of La/SSB mRNA, exon 1 exon 1' and exon 1'' can be detected. In case of exon1' the exon 1 was replaced with an alternative 5' end, moreover the difference between the exon 1' and exon 1'' is only four nucleotides (Tröster 94).

The aim of this study was to determine whether the three Ro/SSA antigen mRNAs and the different splicing variants of La/SSB mRNAs exist in a transformed human keratinocyte cell line (HaCaT). Moreover our goal was to investigate the effect of UVB and estrogen treatment on the expression of Ro/SSA and La/SSB mRNA forms in HaCaT cells. The RNA was isolated from HaCaT cells and was transcribed to cDNA. The methods used to detect the mRNAs forms were PCR.

Inappropriate expression of cytokines is common in autoimmune lesions (La Cava 99). The cytokines, which participate in the regulation of the inflammatory response, can be divided functionally into proinflammatory (IL-1, IL-6, TNF) and anti-inflammatory (IL-1RA, IL-10) molecules (Hurme 98). Interleukin 10 (IL-10) is a pleiotropic cytokine produced by a number of cell types including lymphocytes, monocytes, macrophages and various tumour

cell lines (Burdin 93, Yssel 92). This cytokine was originally described as cytokine synthesis inhibitory factor because of its ability to inhibit the secretion of cytokines from T helper type 1 (Th1) T cell clones (Fiorentino 89). For example, IL-10 inhibits the secretion of tumor necrosis factor α (TNFα), IL-1, IL-6 and IL-12 from monocytes and macrophages (DeWaal Malefyt 91, Fiorentino 91) and interferon γ (IFNγ) and IL-2 from T cells (Sher 91, Taga 92). Effects of IL-10 on cytokine production from Th1 cells and macrophages have led to its classification as a Th2, anti-inflammatory cytokine (Katsikis 95). IL-10 also down-regulates T cell activation by decreasing Major Histocompatibility Complex (MHC) class II expression on antigen presenting cells, but it exerts potent stimulatory effects on B cells by inducing MHC class II expression, cellular proliferation and differentation (Mossmann 94). The IL-10 gene maps to chromosome 1. In the 4 kb sized promoter region several polymorphisms have been described: two microsatellites (dinucleotide repeats) in the 5' flanking region of the gene (Eskdale 95, 96) and three single nucleotide polymorphisms (SNP) at positions -1082 (G to A substitution), -819 (C to T substitution) and -592 (C to A substitution) (Turner 97). These three polymorphisms occur in three putative haplotypes: GCC (Eskdale 95). The IL-10 production is controlled by the G at position -1082, GG genotype is the high IL-10 producing, while AA is the lowest producing genotype in the Caucasian population. The rate of IL-10 production is critical in immune regulation, controlling the balance of inflammatory and humoral responses. Differential expression of IL-10 has been implicated in a number of autoimmune disorders including Sjögren's syndrome.

The objective of our study was to analyse the role of IL-10 nt-1082 promoter polymorphism in the clinical and immunologic characteristics of patients with Sjögren's syndrome. We determined the genotype frequncies by PCR-RFLP method and measured the plasma IL-10 concentrations both in Ss patients and healthy controls.

# II. SUMMARY OF LITERATURE

## IMMUNE SYSTEM

The major role of the immune system is to distinguish the "self structures" from the "non-self structures" and to react properly to their presence. During the evolution two kinds of immune system were developed: the innate/natural/native immunity and the adaptive immunity. Innate immunity is the first line of defense against infections. The mechanism of innate immunity exist even before any encounter with microbes and are rapidly activated by microbes before the development of adaptive immune responses. Innate immunity is the phylogenetically oldest mechanism of defense against microbes and is present in all multicellular organisms. The principal components of this system are physical and chemical barriers (epithelium), phagocytic cells (e.g macrophages), natural killer (NK) cells and blood proteins (complements, etc.), and also cytokines. The three important functions of the native immunity are: 1. it is the initial response to microbes that prevents infection of the host and, in many instances, can eliminate the microbes; 2. the effector mechanisms of innate immunity are often used to eliminate microbes even in adaptive immune responses; 3. innate immunity to microbes stimulates adaptive immune response and can influence the nature of the adaptive responses to make them optimally effective against different types of microbes (Abbas-Lichtman 2003). The components of the innate immunity recognize structures that are characteristic of microbial pathogens and are not present on mammalian cells. It has evolved to recognize microbial products that are often essential for survival of the microbes. The receptors of the innate immune system are encoded in the germline. The innate immune system does not have memory (Abbas-Lichtman 2003, Gergely-Erdei 2000).

The adaptive immune system is stimulated by exposure to infectious agents and increases in magnitude and defensive capabilities with each succesive exposure to a particular microbe. There are two types of adaptive immune responses: humoral and cell-mediated immunity. The main characteristics of the adaptive immunity are: 1. specificity and diversity (ensures that distinct antigens elicit specific responses, enables immune system to respond to a large variety of antigens); 2. memory (leads to enhanced responses to repeated exposures to the same antigens); 3. specialization (generates responses that are optimal for defense against different types of microbes); 4. self-limitation (alows immune system to respond to newly encountered antigens); 5. nonreactivity to self (prevents injury to the host during responses to foreign antigens) (Abbas-Lichtman 2003).

## AUTOIMMUNITY

Adaptive immunity is an important function of the host defense against microbial infections, but immune responses are also capable of causing tissue damage and disease. The self-tolerance is the property of the immune system that ensures that individuals normally do not respond to their own antigens, molecules. Diseases caused by failure of self-tolerance and subsequent immune responses against self or autologous, antigens are called autoimmune diseases. The autoimmunity results from a failure or breakdown of the mechanism normally responsible for maintaining self-tolerance in T cells, B cells or both. The poteintial for autoimmunity exists in all individuals, because some lymphocytes during their development are capable of reacting against self, since they may express receptors specific for self-antigens and many self-antigens are accessible to the immune system (Abbas-Lichtman 2003). Normally tolerance to self-antigens is maintained by a selection process. Loss of this self-tolerance may result from an abnormal regulation, selection of autoreactive lymphocytes.

The major factors that contribute to the development of autoimmunity are genetic susceptibility, hormonal milieu and environmental triggers, such as infections (Abbas-Lichtman 2003, Gergely-Erdei 2000). Most autoimmune diseases are polygenic, and affected individuals inherit multiple genetic polymorphisms that contribute to disease susceptibility (candidate loci are for example: IL-1, 4, 5, 9, 10, 13, SLAM, GM-CSF, TCRα etc.). Most of the genes are not associated with a disease and an individual susceptibility gene may contribute to different diseases. MHC genes, mainly MHC class II genes show the strongest associations with autoimmune diseases (Abbas-Lichtman 2003, Roitt-Brostoff-Male 96). Hormonal influences also play a role in some autoimmune diseases. Most of the autoimmune diseases are more frequent in women than in man. Probably this predominance is the result of the female sex hormonal influence or other gender-dependent factors (Abbas-Lichtman 2003).

Autoimmune diseases may be classified as organ specific or non-organ specific/systemic depending on whether the response is primarily against antigens localized to particular organs or against widespread antigens (Roitt-Brostoff-Male 96, Szegedi 99). In organ-specific diseases autoantibody or T cell responses against self-antigens with tissue distribution is the typical, although the formation of circulating immune complexes composed of self-antigens and specific antibodies are typically produced in systemic diseases such as SLE, Ss, rheumatoid arthritis (RA) (Abbas-Lichtman 2003).

## SJÖGREN'S SYNDROME

Sjögren's (Ss) syndrome is considered a complex disorder. Susceptibility to the disease can be ascribed to an interplay between genetic factors, hormonal milieu and the environment.

Ss is a systemic autoimmune disorder that mainly affects exocrine glands and usually presents as persistent dryness of the mouth and the eyes due to functional impairment of the

salivary and lachrymal glands (Daniels 92). The most important manifestations of the disease are keratoconjuctivitis sicca and oral dryness caused by diminished lachrymal and salivary gland secretory activity. Classically, this glandular dysfunction is associated with progressive lymphocytic infiltration, which leads to the destruction of acinar and ductal epithelial cells and loss of glandular parenchyma. Antibodies against ribonucleoproteins are frequently present in the sera of Ss patients. Some studies suggest that antibodies against muscarinic acetylcholine receptors may have a role in the development of glandular dysfunction (Fox 99). In the absence of an associated systemic autoimmune disease, patients with this condition are classified as having primary Ss (Talal 93).

Patients with Ss have an increased risk of developing lymphomas, in particular B cell non-Hodgkin lymphomas (B-NHL). Previous studies have suggested that malignant lymphomas occur in less than 10 % of patients with Ss. They are usually low grade, lymphomatosus changes occurring more frequently in primary Ss. Patients with persistent glandular swelling, lymphadenopathy and extraglandular disease are at particular risk (Sutcliffe 98).

Sjögren's syndrome occurs worldwide and in all ages. However, the peak incidence is in the fourth and fifth decades of life, with a female to male ratio of 9:1, sometimes the disease has already appeared in childhood (Zeher 90). The primary Sjögren's syndrome is a disease with a prevalence not exceeding 0.6% of the general population. According to previous studies the frequency of this disease can be as high as 1 % in the population above 55 (Zeher 90, Bolstad 2002).

Familial clustering of different autoimmune diseases and co-association of multiple autoimmune diseases in individuals have frequently been reported (Becker 98). It is common for a Sjögren's syndrome proband to have relatives with other autoimmune diseases (approximately 30-35%). Most often this is Ss (12 %), thyroid disease (14 %), RA (14 %) or

SLE (5-10 %) (Reveille 84, Reveille 92, Tanaka 2001). Clustering of non-major histocompatibility complex (MHC) susceptibility candidate loci in human autoimmune diseases supports a hypothesis that, in some cases, clinically distinct autoimmune diseases may be controlled by a common set of susceptibility genes (Heward 97).

The polymorphic MHC genes are the best documented genetic risk factors for the development of autoimmune diseases overall (Nepom 93, Merriman 95, Tomlinson 95). The most relevant MHC complex genes are the class II genes, specifically the human leukocyte antigen (HLA)-DR and HLA-DQ alleles (Reveille 92). Patients of different ethnic origins exhibit different HLA gene associations (Kang 93). In Caucasians of northern and western European backgrounds, including North American Caucasians, Sjögren's syndrome is associated with the haplotypes B8, DRw52 and DR3. The increased frequency of HLA-B8 was presumably due to an association with the HLA class II allele HLA-DRB1*03. All of the haplotypes are in strong linkage disequilibrium, resulting in certain difficulties in establishing which of the genes contains the locus that confers the risk. Apparently, the HLA haplotype may influence the severity of autoimmune disease. It has been claimed that Sjögren's syndrome patients with DQ1/DQ2 alleles have a more severe autoimmune disease than do patients with any other allelic combination at HLA-DQ (Kerttula 2003), and the DR3-DQ2 haplotype has been indicated as a possible marker for a more active immune response in Finnish patients with Sjögren's disease (Kacem 2001). HLA class II allele association have been reported to differ among antiRo/SSA positive subjects according to the presence or absence of coexisting antiLa/SSB (Miyagawa 98). Distinct HLA haplotypes have been associated with certain degrees of autoantibody diversification in Ss (Rischmueller 98). Autoantibodies to Ro/SSA and La/SSB have been found to be associated with DR3, DQA and DQB alleles (Bolstad 2001, Nakken 2001, Fei 91). A stronger correlation has been found between anti-Ro/SSA autoantibodies and DR3/DR2 than that with the disease itself (Bolstad

2001, Arnett 89, Hamilton 88, Wilson 84). In Japanese persons HLA class II allele association has been reported to differ among anti-Ro/SSA-positive individuals according to the presence or absence of coexisting anti-La/SSB (Miyagawa 98).

Cytokines serve to mediate and regulate immune and inflammatory responses, and have been implicated in the pathogenesis of a variety of autoimmune diseases, including Sjögren's syndrome. Both human and animal studies indicate the involvement of IL-10 in the pathogenesis of primary Sjögren's syndrome (Halse 99) and mice transgenic for IL-10 develop a Fas-ligand-mediated exocrinopathy that resembles Sjögren's syndrome (Saito 99). A recent study described an association between primary Sjögren's syndrome and IL-10 promoter polymorphisms in a cohort of Finnish individuals, and a specific haplotype was found to correlate with high plasma levels of IL-10 (Hulkkonen 2001). Conversely, no association was found for IL-10 promoter polymorphism and primary Sjögren's syndrome or the presence of Ro autoantibodies in an Australian cohort of primary Sjögren's syndrome patients (Rischmueller 2000). The IL-1 receptor antagonist regulates IL-1 activity in inflammatory disorders by binding to IL-1 receptors and thus inhibiting the activity of IL-1. The human IL-1 receptor antagonist gene (IL1RN) has a variable allelic polymorphism within intron 2 as a result of variation in number of an 86-base-pair sequence repeat (Magnusson 2001). An increased frequency and carriage rate of the IL1RN*2 allele has been found in primary Sjögren's syndrome (Perrier 98). No statistically significant association can be ascribed to tumour necrosis factor-α and primary Sjögren's syndrome (Jean 98).

## AUTOANTIBODIES IN SJÖGREN'S SYNDROME: Ro/SSA and La/SSB

Dysregulation of B cells and hypergammaglobulinemia are prominent recognised features of the Sjögren's syndrome. Elevated levels of circulating immunoglobulins usually

contain several specificities for self-molecules of both organ-specific and non-organ-specific targets, the most common autoantibodies being: rheumatoid factor (RF), antiRo/SSA and antiLa/SSB. Other autoantibodies in Ss are anti-vimentin, anti-α-fodrin, anti-histone, etc. These antibodies have a high affinity and show characteristics of an antigen-driven, T cell-dependent immune response, belonging primarily to the IgG1 and IgG3 subclasses of immunoglobulin, although recent studies have described both IgA and IgM specific for the Ro and La proteins (Rose 98).

The aggregates of lymphocytes form around the intralobular ducts and in the surrounding acinar epithelium are the part of the histopathological development of the Ss. The majority of the lymphocytes are T cells, 10-20 % of the infiltrating lymphocytes are B cells. These cells are often found at the margins of the focal infiltrates or as a small conglomerates throughout the parenchyma, and the number of plasma cells which produce antibodies of IgG and IgM isotypes is increased but the number of IgA producing cells can be reduced. The location of the B, T cells and plasma cells indicate that the B cells are driven to activation and autoantibody production with the help of the T cells in the area of inflammation (Speight 94).

The role of autoantibodies in the autoimmune process is not fully understood and it is unknown whether the antibodies reacting with the intracellular targets in vivo cause inflammation. The La antigen in salivary gland epithelial cells has been shown to re-localise from nucleoli to nucleoplasma, cytoplasma and to the membrane surfaces of the acinar cells in Ss patients (DeWilde 96). In fact, some authors suppose that Ro and La antigens could be detected on the cell membrane as consequence of viral infection (Baboonian 89), high concentration of 17-β-estradiol (Ciocca 95) or myocardiocytes apoptosis (Buyon 98, Miranda 98). This implies that the antigens stimulating the antibody producing cells which are present in the salivary glands, and that the B cells can be stimulated by these antigens to produce autoantibodies which contribute to the salivary gland inflammation and destruction.

AntiRo/SSA and antiLa/SSB antibodies were originally described as two precipitating antibody specificities reacting with antigens contained in salivary and lachrymal glands' extract of Ss patients (Jones 58, Anderson 61). These autoantibodies are found both in the sera of patients with Ss, SLE and RA, or overlaps of these diseases. A strong association between antiRo/SSA and antiLa/SSB antibodies has been observed. AntiRo/SSA antibodies can be found alone in many sera, while antiLa/SSB antibodies usually accompanied by antiRo/SSA. These autoantibodies are detected more frequently in patients with rheumatoid factor, polyclonal hypergammaglobulinemia (Alexander 83) independently from the autoimmune disease. Some investigators suggest that the titres of antiRo/SSA antibodies fluctuate during the course of the Ss and SLE, reporting a correlation with disease activity (Praprotnik 99, Scopelitis 80, Derksen 92, Wahren 98). The presence of the antiRo/SSA and antiLa/SSB autoantibodies is one of the items in the classification criteria suggested by the European Community Study Group on Diagnostic Criteria for Ss (Vitali 2002).

The association between the antiRo/SSA and antiLa/SSB and disease subsets, suggests that these antibodies may be involved in the pathogenesis of the tissue damage. Several investigators found that these antibodies are produced locally in Ss patients's inflamed salivary glands (Tengner 98, Penner 82), and Maddison et al. eluted antiRo antibodies from kidneys of patients with SLE, moreover the presence of this autoantibody is associated with photosensitive symptoms (Maddison 79). AntiRo and antiLa antibodies were studied in mothers whose children had neonatal lupus erythematosus (NLE) (Tseng 96, Franceschini 99). In the pathogenesis of NLE the antiRo and antiLa antibodies are involved, these antibodies can cross the placenta and can cause NLE, which is characterised by transient skin rash and a cardiac conduction defect (Buyon 93). The tissue injury is related to the expression of Ro and La antigens in foetal cardiac tissue, particularly located at the surface of the myocardial fibres of the foetus (Deng 87, Taylor 86, Tseng 97). Furthermore, human IgG

containing antiRo/SSA can induce transmembrane repolarisation in neonatal rabbit hearts (Alexander 92, Garcia 94) by interfering with the calcium-channel activity (Boutjdir 97, 98, Xiao 2001). Interestingly UVB irradiation of keratinocytes increases the expression of Ro antigen on the cell surface (Furukawa 90), enhancing the possibility of direct injury of the skin cells by antiRo antibodies (Golan 92).

The Ro antigen is constituted by two different proteins of 52 kDa and 60 kDa molecular weight proteins and there is a third protein, which is also called calreticulin, the 46 kDa molecular weight Ro. Calreticulin is a multifunctional protein that acts as a major calcium-binding protein in the lumen of the endoplasmic reticulum. It is also presents in the nucleus, suggesting that it may have a role in transcription regulation. Calreticulin binds to the synthetic peptide, which is almost identical to an amino acid sequence in the DNA-binding domain of the superfamily of nuclear receptors. McCauliffe et al. showed that calreticulin binds to antibodies in certain sera of SLE and Ss patients which contain antiRo/SSA antibodies, and that is located in the endoplasmic and sarcoplasmic reticulum where it may bind calcium. The calreticulin locus is located in the region 19pter-p13.2 (McCauliffe 90).

Lerner et al. showed an association of Ro antigens with small RNAs and refered this antigen system as Ro ribonucleoproteins (RoRNP) (Lerner 81) (Fig 1.). This RNP complex contains at least two proteins, Ro 60 kDa and Ro 52 kD. The 60 kDa binds to one of four 83-112 base long RNAs termed human cytoplasmic RNAs (hYRNA). Human cells contain four hYRNA (hY1, hY3, hY4, hY5RNA) they are present in about $1\text{-}5x10^5$ copies per cell (Hendrick 81, Wolin 84). The hYRNAs reside in the cytoplasm, while the Ro proteins are present in the nucleus as well as in the cytoplasm. The biological functions of this complex are not known, but results suggesting the Ro 60 kDa protein may be involved in a discard pathway for detective 5S rRNA precursors have been presented.

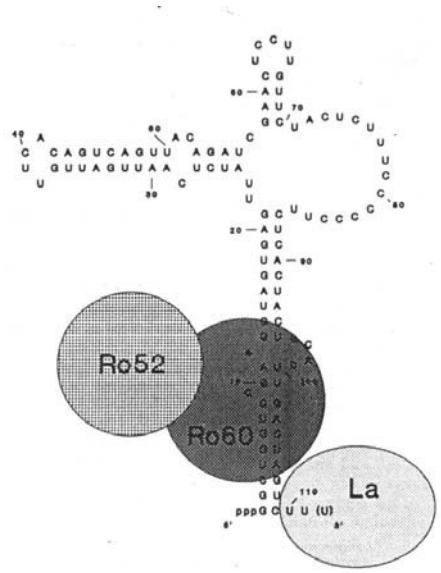


**Fig. 1.** This figure demonstrates an example of the Ro/La RNA complex. (Lerner et al. 81)

In humans the Ro 52 kDa protein is encoded by a gene located on chromosome 11p15.5 (Frank 93, Grölz 94), and shows zinc finger and leucine zipper domains without a specific RNA binding site (Chan 91).

The 60 kDa Ro protein is encoded by a 1.8 kb gene, located on chromosome 1q31 (Grölz 94). It has a zinc-finger motif which is an RNA binding domain (Deutscher 88). The amino acid sequences of each Ro proteins are quite different, and the 46 kDa and the 60 kDa antigens have limited homology with foreign polypeptides, raising the possibility that an immune response which originally attacks a foreign protein can be directed against the self proteins (Scofield 91).

The autoepitopes of 60 and 52 kDa Ro proteins have a different conformation dependence. In the 52 kDa proteins most sera recognise only linear epitopes in the leucine zipper site, not expressed on the surface of the native molecule. The epitopes recognised by anti 60 kDa Ro/SSA antigen are highly conformational (Itoh 92a, b, Boire 89, 91) and with the denaturation of the protein the antibody binding to the 60 kDa antigen is lost. AntiRo 52 kDa antibodies immunoprecipitate the Ro-RNP complex only in presence of anti 60 kDa

protein. This hypothesis was confirmed by the evidence of molecular interaction between leucine-zipper region of the 52 kDa Ro and three peptides of 60 kDa Ro, the 52 kDa Ro/SSA bind to the Ro-RNP complex by aminoacidic interaction (197-207 linear epitope), and together all these peptides constitute a tertiary epitope (Kurien 2001). It has been demonstrated that ultraviolet B (UVB) radiation, estrogen treatment and some viral infections result in translocation of the Ro/SSA antigens to the cell surface, where they are available to bind circulating Ro/SSA autoantibodies (LeFeber 84, Furukawa 90, 88, Tesar 86). Similar results have been reported with UVB radiation for the La/SSB antigen (Bachmann 90).

The La/SSB antigen, which is located primarily in the nucleus, is a single phosphorilated protein transiently associated with all RNA polymerase III transcripts (5S rRNA, tRNA, some 4.5S RNA, as well as a portion of the U1- and U6 snRNA) the hYRNAs, through binding of its uridine-rich 3' end, which is transcribed during the transcription termination step (Hendrick 81, Madore 84, Rinke 85, Pruijn 90). These oligoU-tails were shown as a binding site for the La protein (Stefano 84). The 46–50 kDa La/SSB protein carries autoantigenic determinants, and it has been suggested that La/SSB participates in the synthesis, maturation and nuclear export of this class of RNA molecules. Recently, it has also been shown that in lymphocytes of patients with Sjögren's syndrome there are three different splicing variants of La/SSB mRNA (Fig. 2) (Tröster 94). Five La cDNAs were isolated when a cDNA library was made from peripheral blood lymphocytes (PBLs) of a patient with Ss (Tröster 94). In two of these five La cDNAs the exon 1 was replaced with an alternative 5'end. This is an alternatively spliced transcript of the La gene. And an additional promoter site was identified in the intron between the exon 1 and exon 2, which served as initiation site for transcription of the alternative exon 1'.

The exon 1' La mRNA form has an unusual 5' terminus. It contains GC-rich regions, an oligoU-tail of 23 uridine residues and encodes for three upstream open reading frames

(ORF1, ORF2 and ORF3). The ORF1 encodes for a putative peptide of 5.4 kDa. It was interrupted from the La protein reading frame by two stop codons. The ORF2 and ORF3 were not in the reading frame of the La protein. Qualitative and quantitative analysis of expression of the exon 1 and exon 1' La mRNAs showed that both La mRNA forms represented finally processed abundant cytoplasmic mRNAs. Exon 1 to exon 1' La mRNAs were expressed at ratios between 1:1 and 1:5 (Hilker 96, Bachmann 96).

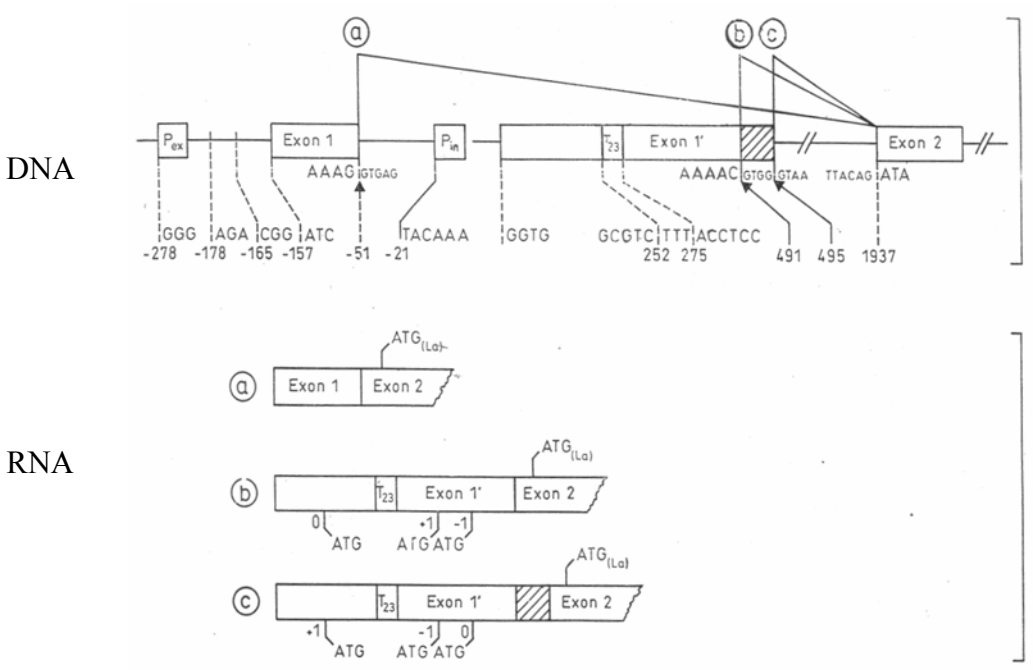


**Fig. 2:** The three different splicing variants of La mRNA. The DNA is alternatively spliced and three forms of mRNA are expressed (*a* exon 1, *b* exon 1' *c* exon 1''). The ATG (in exon 2) represents the start of the protein coding section but other alternative start sites can be found in the other two forms (*+1* and *–1* indicate one nucleotide alteration from the correct reading frame) (Tröster et al. 1994)

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Within the intron between exon 1 and exon 1' there have been found some putative transcription factor binding sites. For example TFIID binding site and other transcription factors binding sites, which could be involved in the regulation of differential expression of the two types of La mRNAs. These transcription factor binding sites include common transcription factors such as SP1, AO1, AP2 and NF-κB element 218 nt downstream of the exon 1, as NF-kB is known to be activated by UV irradiation, dsRNA, acute phase proteins,

including some interleukins, interferons, etc. (Schreck 92). The NF-kB element also exists in retroviruses, viral latency and development of autoimmunity.

One of the La mRNA forms contains a frame shift mutation in the hot spot region of the La gene. The encoded mutant La protein lacks the C-terminal La domain and thereby the nuclear location signal (NLS). The frameshift mutation causes a premature stop codon in the La protein reading frame. Thus the mutant exon 1' La mRNA could encode for a C-terminally truncated mutant La peptide of 29 kDa (Bachmann 96) (Fig. 3.).

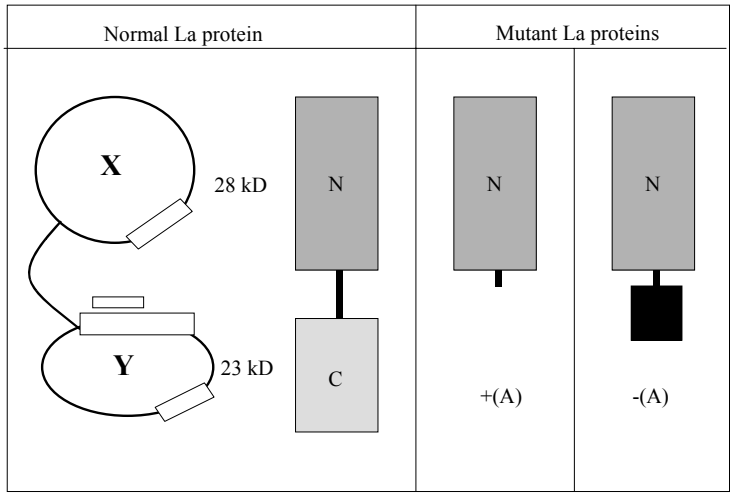


**Fig. 3.:** The protein contains two domains, X 28/29 kDa N-terminal and Y 23/24 kDa C-terminal domain which are associated by a protease-sensitive chain (130 aminoacid). The X domain includes the oligoU-tail, the Y includes a NLS and an ATP-binding site (Bachmann et al. 1996)

In summary, genomic data show that the mechanism leading to the alternative form of La mRNA includes both a promoter switching and an alternative splicing event (Tröster 94).

## CYTOKINES IN AUTOIMMUNITY: Interleukin-10

Cytokines are soluble, non-antigen specific glycoproteic local mediators that determine the types of cells recruited to a lesion, modulate the activation state of

inflammatory cells and participate in the noncognate intercellular communication between immunocompetent cells. Innappropriate expression of cytokines is a common finding in any autoimmune lesion (Gergely-Erdei 2000, Theofilopoulos 99). Cytokines regulate immune and inflammatory reactions as well as haematopoiesis, activate the immediate non-specific antimicrobial effectors, macrophages, natural killer (NK) cells, and they are required for the initiation of antigen-specific immune responses, although a prolonged or too strong immune reaction is made responsible for the adverse effects of infectious and autoimmune disease (Hurme 98). Cytokine actions may be local and systemic. Most cytokines act close to where they are produced, either on the same cell that secretes the cytokine (autocrine) or on a nearby cell (paracrine), and they may enter the circulation and act at a distance from the site of production (endocrine). Receptors of cytokines often bind their ligands with high affinities, and the levels of the cytokines in the plasma are usually low, (Abbas-Lichtman 2003, Gergely-Erdei 2000, Hurme 98), the optimal levels can guarantee the successful eradication of an invading microbe and to avoid excessive damage to the host (Hurme 98).

The cytokines can be classified into three main functional categories based on their biologic actions:

1. mediators and regulators of innate immunity are produced mainly by mononuclear cells in response to infectious agents (IL-1αβ, IL-6, TNF-α, chemokines, IL-12, IL-10, etc.)
2. mediators and regulators of adaptive immunity are produced mainly by T lymphocytes in response to specific recognition of foreign antigens (e.g. IL-2, IL-4, IL-5, IFN-γ, TGF-β)
3. stimulators of hematopoiesis are produced by bone marrow stromal cells, e.g. leukocytes, stimulate the growth and differentiation of immature leukocytes (IL-3, IL-7, GM-CSF, etc.) (Abbas-Lichtman 2003).

The cytokines, which participate in the regulation of the inflammatory response (1. category), can be divided functionally into proinflammatory (IL-1, IL-6, TNF) and anti-inflammatory (IL-1RA, IL-10) molecules. After microbial induction, the proinflammatory molecules are generally induced first and very rapidly (within the first minutes or hours of the invasion), and this is followed by the production of the anti-inflammatory cytokines (Hurme 98).

Cytokines are involved in every stage of the autoimmune process. They recruit inflammatory cells to autoimmune lesions by chemoattraction and modulate the inflammatory process and the tissue damage. Some cytokines can enhance T cell mediated cytotoxicity, facilitate loss of self-tolerance, and activate autoreactive T lymphocytes owing to the fact that some cytokines can induce aberrant expression of MHC molecules on target cells and favour hyperexpression of target antigens (Feldmann 93).

Autoreactive T cells and autoantibodies are components of the normal/healthy immune system. The autoimmunity is prevented by regulatory mechanisms (King 97), loss of the balance of immune homeostatic mechanisms may result in abnormal immune responses that can lead to autoimmunity. The presentation of autoantigens could occur upon aberrant expression of MHC antigens on inappropriate cells, and it would be responsible for initiating pathogenic autoreactivity and autoimmune reactions for example (Bottazzo 83).

Genetically determined patterns of cytokine secretion may contribute to autoimmune disease susceptibility. Lots of study have reported abnormal patterns of cytokine expression in various autoimmune conditions, including RA, multiple sclerosis, SLE, etc. (De Carli 94), but it is difficult to define whether the imbalance of cytokine expression contributes to the pathogenic process or other abnormal process may be involved in it (La Cava 99).

The presence of specific cytokines in the milieu at the site of autoimmune attack may be crucial in determining the outcome of the autoimmune response at the target organ. Since the cytokines are produced by a number of cell types and manifest pleiotropic effects, the

cytokines may contribute a local immune response and recruit pathogenetic cells that initiate, maintain the damage at the site of the autoimmune aggression. $CD4^+$ T cell subsets produce specific cytokines, which can specifically sustain the local imbalance of cytokine production (La Cava 99). $CD4^+$ T cells can be categorized into three functional groups, T helper type 1 (Th1), Th2 and Th0 based on their profile of their cytokine production. Th1 cells produce IFNγ, IL-2, IL-12, TNFβ, Th2 cells produce IL-4, 5, 6, 10 and IL-13, Th0 cells produce both IL-2 and IL-4. The cytokines of Th1 and Th2 cells are mutual antagonists (Mosmann 89). Any imbalance between the Th1 and Th2 responses may lead the development of abnormal immune response and/or autoimmune diseases (La Cava 99). Animal models suggest that Th1-type cytokines are involved in the beginning of organ-specific autoimmune diseases (Liblau 95). In contrast, Th2-type cytokines are involved in systemic autoimmune diseases (Goldmann 91). In this group a more heterogenous cytokine pattern rather than a typical Th profile is usually detectable, for example in Ss the IL-1 and 6, and TNFα and IFNγ are upregulated in salivary glands (Oxholm 92).

Interleukin 10 (IL-10) is a 36 kDa homodimeric, pleiotropic cytokine produced by a number of cell types including lymphocytes, monocytes, macrophages and various tumour cell lines (Burdin 93, Yssel 92, Turner 97, Moore 93). This cytokine was originally described as cytokine synthesis inhibitory factor because of its ability to inhibit the secretion of cytokines from T helper type 1 T cell clones (Fiorentino 89). IL-10 which is produced by T and B lymphocytes and macrophages has different effects in different cell types (Moore 93). For example, IL-10 inhibits the secretion of tumour necrosis factor α (TNFα), IL-1, IL-6 and IL-12 from monocytes and macrophages (De Waal Malefyt 91, Fiorentino 91) and interferon γ (IFNγ) and IL-2 from T cells (Sher 91, Taga 92). IL-12 is a critical stimulus for IFNγ secretion, so the inhibition of IFNγ secretion by T cells an indirect effect of IL-10 suppression of IL-12 production in macrophages and dendritic cells (D'Andrea 93, Abbas-Lichtman

2003). The effects of IL-10 on cytokine production from Th1 cells and macrophages have led to its classification as a Th2, anti-inflammatory cytokine, although some studies have found that IL-10 were produced by a significant number of Th1 as well as Th2 cell clones (Katsikis 95).

IL-10 also down-regulates T cell activation by decreasing MHC class II expression on antigen presenting cells, but it exerts potent stimulatory effects on B cells by inducing MHC class II expression, cellular proliferation and differentation (Mossmann 94), whereas this cytokine blocks MHC class II expression (De Waal Malefyt 91) and B7 expression (Ding 93) on the surface of monocytes and reduces the capacity of the monocytes to activate T cells. IL-10 reduce MHC class I expression on the surface of cytotoxic T cell (CTL) target cells in a dose dependent manner, leading to a decrease in CTL-mediated lysis (Matsuda 94).

The IL-10 gene maps to the junction of 1q31-q32 (Eskdale 97), contains five exon and four introns (Gergely-Erdei 2000). In the IL-10 gene there are several types of polymorphisms and varying numbers of dinucleotide repeats, microsatellites and base exchanges. These alleles are functionally different (Hurme 98, Semsei in press) (Fig. 4.).

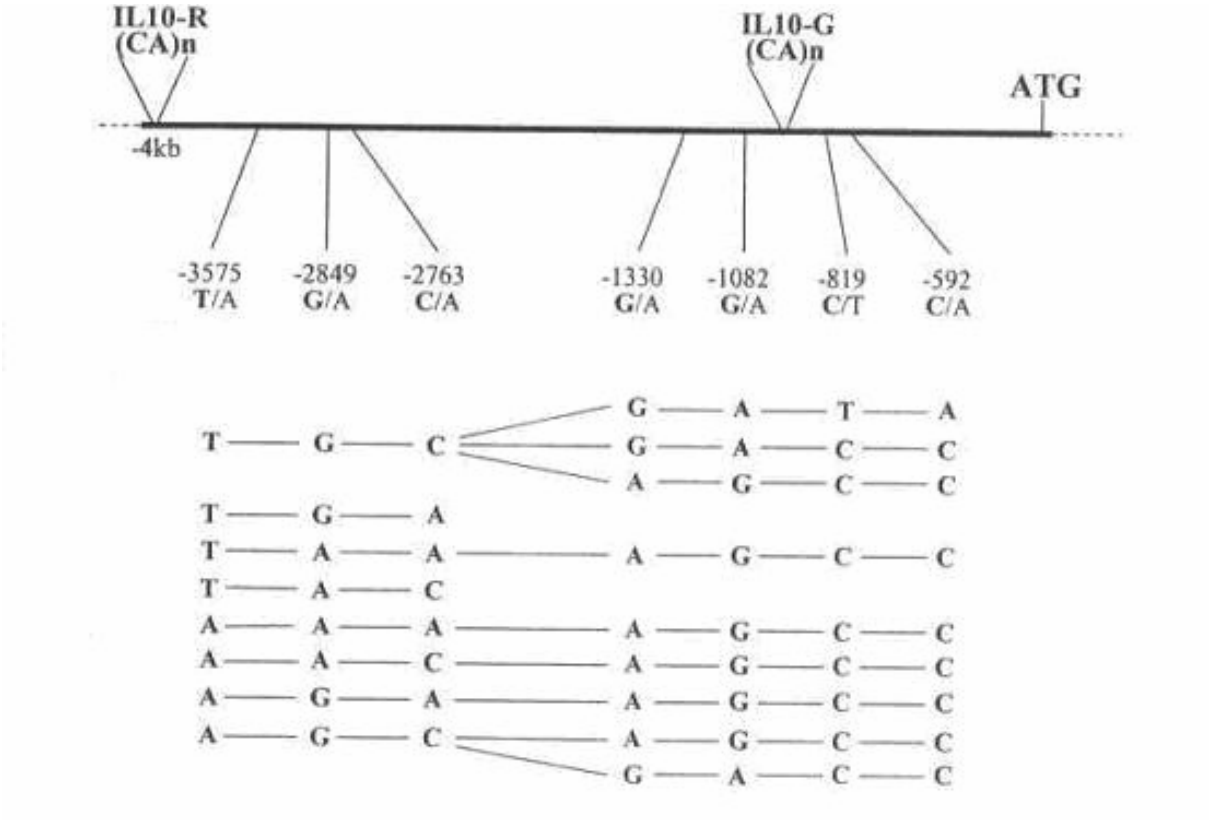


**Fig. 4.:** The promoter region of the IL-10 gene. The different SNPs determine several haplotypes. (Gibson et al. 2001)

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Eskdale et al. (Eskdale 95,96) determined two microsatellites, CA repeats in the promoter region of the IL-10 gene, IL10.G and IL10.R in the 5' flanking region. The IL10.R microsatellite lies between -4004 and -3978 in the 5' flanking region of the IL-10 gene. It is approximately 2800 bp upstream of the IL10.G microsatellite which lies between -1198 and -1151. They identified 16 different alleles, allele 1 has the smallest, and allele 16 has the biggest size. The most frequent was the allele 9 (50.5 %). The observed total heterozygosity was 71.6 % (Eskdale 95). They identified three alleles at the locus of IL10.R, whose sizes are 114, 116 and 118 bp. They termed them as IL-10R.2, IL10.R3 and IL10.R4, since other studies revealed the presence of two additional alleles. One was smaller than IL10.R2, so it is termed as IL10.R1, and one was larger than IL10.R4, thus termed IL10.R5. From the five alleles the IL10.R4 is the most rare, while IL10.R2 and IL10.R3 are both common in the

examined population. The observed total heterozygosity was 50% and these were mainly from IL10.R2/3 heterozygotes. They examined the relationship between the alleles of IL10.G and IL10.R loci. They found four significant associations: IL10.G11/R2, G13/R2, G9/R3, G10/R3. The data suggest that these alleles may usually be carried on the same strand of DNA and constitute haplotypes (Eskdale 96).

Turner et al. (Turner 97) identified three single nucleotide polymorphisms (SNP) or single base pair substitutions in a 1132 bp region (+17 from the transcriptional start site to -1115) of the IL-10 promoter by single-strand conformational polymorphism (SSCP) analysis and direct sequencing. The three SNPs are: -1082 G/A, which lies within an ETS-like recognition site (Kube 95) and may affect the binding of the transcription factor which acts as a negative regulator of IL-2 production (Romano-Spica 95); -819 C/T and -592 C/A. These three polymorphisms exhibit strong linkage disequilibrium and occur in three putative haplotypes: GCC (G at position -1082, C at position -819 and C at position -592), ACC and ATA (Eskdale 95). In the examined control group the frequency of the G allele at -1082 nt was 0.51, of the A allele was 0.49, but at position -819 and -592 the frequency of the C allele was 0.79 since the T (nt-819) and A (nt-592) allele frequency were 0.21 and the GCC haplotype was the most frequent (0.51). Following ConA stimulation the plasma IL-10 levels were measured by ELISA. The controls who were negative for A at position -1082, showed significantly higher IL-10 protein production compared to subjects who were A allele positive. So the ability of individuals to produce high levels of IL-10 is evidently controlled by a G at position -1082, as this variant is found in the highest producers. Several studies found that the GCC haplotype is more frequent than the other two haplotypes. Reynard et al. examined the population of South-East England. The frequency of the GCC was 0.526, while the frequency of the ACC and ATA were lower (0.31 and 0.164) (Reynard 2000). When these frequencies were compared to the results of a Manchester population study (Perrey 98) there

were not found any significant differences between the haplotype frequencies (GCC 0.494, ACC 0.288, ATA 0.218). However, published frequencies for a population from Southern China (Mok 98) did differ significantly from those found in Perrey's and Reynard's study. In the Southern Chinese population the GCC haplotype frequency was significantly lower (0.02), and the ATA haplotype frequency was higher (0.64) than in the UK studies. So the high IL-10 producer GCC haplotype is more common in the Caucasian population, while in the Oriental population the -1082 A allele was observed at a significantly higher frequency (Reynard 2000).

Hulkkonen et al. (Hulkkonen 2001) analyzed the IL-10 plasma levels and polymorphisms of the IL-10 gene in Sjögren's syndrome patients and compared the results with healthy control subjects. They found that the frequency of the GCC haplotype was increased and the frequency of the ACC haplotype was decreased in pSs. The GCC haplotype was associated with high plasma levels in Ss patients which was in agreement with the results observed by Turner et al. (Turner 97). Llorente et al. also found elevated levels of IL-10 mRNA and protein expression in peripheral blood mononuclear cells of Ss patients (Llorente 94).

Font et al. (Font 2002) also investigated the IL-10 polymorphisms in Ss patients. They also found an increased GCC haplotype (0.484) and a lower ACC haplotype frequency (0.246) in Ss patients compared to the healthy controls. Font et al. found a higher frequency of the GCC/ATA genotype and a significant decrease in the prevalence of the ACC/ACC genotype in Ss patients. The other major finding of this study was that in Ss patients who were carriers of the IL-10 GCC haplotype, the disease onset was earlier (48.06 year↔57.53 year). It is may be a prognostic significance in patients, because the risk of lymphoma development increases in patients with early disease onset (Ramos-Casals 98). No differences were observed in the immunological and clinical features in Ss patients according to the IL-10

genotypes. Rischmueller et al. (Rischmueller 2000) also did not find significant differences in the analysis of analytical, immunological and clinical features, however they did not find significance difference in view of haplotype frequencies. The results suggest that the IL-10 promoter polymorphisms may be an important component of the genetic background of Ss.

Origuchi et al. (Origuchi 2003) analyzed the correlation between IL-10 gene promoter polymorphisms and clinical manifestations in Japanese Ss patients. They found that the GCC haplotype, which is predominant in white, caucasian subjects, was less common in the Japanese population, while the ATA haplotype frequency was significantly higher in Japanese people. In Ss patients the number of the ACC haplotype (0.18) carrier was significantly lower compared with control subjects (0.29), while the frequency of the ATA haplotype was increased in patients. The age at onset of ACC haplotype non-carriers was significantly lower than that of ACC haplotype carriers. They did not find any association between the haplotypes and Ss features. Their results suggested that the presence of the ATA haplotype and the absence of the ACC haplotype were associated with an increased susceptibility to pSs in Japanese people.

# III. THE AIM OF THIS STUDY

Anti-Ro/SSA and anti-La/SSB antibodies were originally described as two precipitating antibody specificities reacting with antigens contained in salivary and lachrymal glands' extracts of patients affected by Sjögren's syndrome (Ss) (Anderson 61). Anti-Ro/SSA antibodies are the most prevalent specificity among many autoimmune diseases. SLE (Maddison 81), homozygous complement deficiency SLE (C2 and C4) (Provost 83, Tappainer 82), Ss/SLE overlap syndrome (Provost 98), subacute cutaneous LE (SCLE) (McCauliffe 97), neonatal lupus (Buyon 93) are frequently observed in association with anti-Ro/SSA. In contrast, anti-La/SSB antibody is more closely associated with Ss. A strong association between anti-Ro and anti-La antibodies has been observed. Anti-Ro/SSA antibodies can be found alone in many sera, while anti-La/SSB antibodies are usually accompanied by anti-Ro/SSA (Alexander 83). Sjögren's syndrome presents usually in middle-aged women, the role of the hormonal milieu is presumable in the etiopathogenesis of this disease (Abbas-Lichtman 2003). Sex hormones influence both humoral and cell-mediated adaptive immune response, and estrogen is one of the potential factors of this immunologic dimorphism (Lahita 95, Bateman 89). The effect of estrogen has been suggested to be responsible for the female predominance in autoimmune diseases such as SLE and Ss (Lahita 82, Daniels 94). The role of estrogen metabolism abnormalities was also supported by clinical and experimental results (Lahita 79). It was demonstrated that the binding of Ro/SSA and La/SSB autoantigens was increased to the surface of keratinocytes after estradiol treatment. However it was noticed that the effect of UVB irradiation also results in surface presentation of the Ro and La antigens (Furukawa 90, Bachmann 95), so the circulating antibodies can bind to these surface antigens, enhancing the possibility of direct injury of the skin (Golan 92). HaCaT cells were chosen because the binding of the Ro and La antigens to the cell surface can be detected only on fast

reproducing, non-differentiated cells, and this cell-line may be considered as an in vitro model of the proliferative basal keratinocytes. We aimed to find the answer for the following questions:

- What is the basal level of the Ro/SSA and La/SSB antigens mRNAs in HaCaT cells?
- Is there any effect of the UVB irradiation and 17-β-estradiol on the expression of the levels of the Ro/SSA and La/SSB antigens mRNAs?
- Is there any association between the mRNA expression of Ro/SSA and La/SSB autoantigens and the role of the estradiol and UVB irradiation in the disease susceptibility and development?

Several studies reported from an association between the IL-10 gene polymorphisms and the disease susceptibility and onset (e.g. Eskdale 97, Lazarus 97, Hajeer 98, Hulkkonen 2001, Font 2002). The importance of IL-10 polymorphisms was emphasized in connection with some autoimmune disease, for example autoimmune hepatitis, autoimmune thyroid disease, etc. Beside autoimmune diseases many malignant diseases can be connected with IL-10 promoter polymorphisms such as Hodgkin-lymphoma and Melanoma (Herbst 96, Becker 94). The IL-10 production is genetically encoded, and the IL-10 may be an important factor in the study of autoimmune and malignant diseases.

A study of the IL-10 promoter nt-1082 polymorphism was proposed in order to be able to answer the following questions:

- Is there any association between the IL-10 gene nt-1082 polymorphism and the disease?
- Is there any association between the IL-10 genotypes and the plasma IL-10 levels?

- Is there a prognostic role of the IL-10 genotype and plasma levels in the onset and development of the disease?
- Is there a prognostic role of the different Ss features according to the IL-10 genotype and Il-10 levels in the disease procession?

# IV. MATERIALS AND METHODS

## mRNA expression of Ro/SSA and La/SSB autoantigens in HaCaT cells

***Cell culture.*** Cells of the transformed human keratinocyte cell line, HaCaT, were grown in DMEM (Sigma), supplemented with 10% heat-inactivated foetal bovine serum, antibiotic-antimycotic solution (100 U penicillin, 100 U streptomycin, 2.5 mg fungizone, Gibco), 4 mM L-glutamine (Gibco) at 37 °C and 95% relative humidity in an atmosphere containing 5% $CO_2$ in 60-mm plastic dishes. The medium was changed every 2–3 days.

***Estradiol stimulation.*** Water-soluble 17-β-estradiol (Sigma) solution was prepared freshly prior to each experiment. HaCaT cells were cultured to 50% confluence, then estradiol was added to a final concentration of $10^{-8}$ M. HaCaT cells were incubated with $10^{-8}$ M 17-β-estradiol for 12, 24, 48 and 72 h. The effect of estradiol at a concentration of $10^{-7}$ M was also evaluated but we did not find any significant difference between the effects of the two concentrations. However, according to Wang and Chan (Wang 96) more significant changes at a concentration of $10^{-8}$ M regarding the mRNA levels of Ro could be expected.

***UVB irradiation.*** Before irradiation of subconfluent HaCaT cells the medium was replaced with phosphate-buffered saline (PBS), which was free of any photoactive compound.

At least one dish was not exposed to UV radiation, but was handled in the same fashion as the irradiated cells. The remainder was exposed to UVB, produced by a Saalmann Multitester lamp (Saalmann, Herford, Germany), at a dose of 200 J/$m^2$. After irradiation the PBS was replaced with DMEM and the cells were harvested after 12, 24, 48 and 72 h.

***Isolation of RNA and reverse transcription to cDNA.*** The HaCaT cells were mixed in Trizol (Sigma) using an Ultraturrax. Further processing was based on the method of Chomczinsky (Chomczinsky 93) in which a mixture of guanidinium-thiocyanate-phenol is used for the isolation of RNA. The RNA was further purified using guanidine hydrochloride (Semsei 89). The cDNAs were transcribed from the isolated total RNA using a Superscript reverse transcriptase system (GIBCO).

Isolation of RNA:

- homogenation with Ultraturrax, 1 ml Trizol, 10 sec
- incubation at RT, 5 min and adding 0.2 ml chloroform to the sample, vortex 15 sec → to ice 3 min
- centrifugation 11000 rpm, 15 min
- transferring the upper phase to another Eppendorf tube
- adding to it one volume isopropanol, 10 min on ice
- centrifugation 14000 rpm, 30 min
- washing with 1 ml 75 % ethyl-alcohol
- centrifugation 14000 rpm, 5 min
- dissolving the RNA in 10 µl TE
- determination of the RNA concentration by spectrophotometer (1 $OD_{260}$= 37.5µg/ml)

cDNA synthesis:

- reaction mix is the following:

| | |
|---|---|
| distilled water | 11-x µl |
| primer $(oligodT)_{12-18}$ | 1 µl |
| RNA (3 µg conc.) | x µl |
| TOTAL | 12 µl |

- incubation at 70 ˚C, 10 min
- 1 min on ice
- adding to the sample:
    - 2 µl 10x synthesis buffer
    - 1 µl 10 mM dNTP
    - 2 µl 0.1 mM DTT
    - 2 µl 25 mM $MgCl_2$
- incubation at 25 ˚C, 30 sec
- adding 1 µl SuperScript reverse transcriptase (200U/µl)
- PCR : 29 ˚C, 15 sec; 42 ˚C, 1 min; 70 ˚C, 15 sec
- incubation at 37 ˚C, 15 sec
- adding to the sample 1 µl RNase-H (2U/µl)
- incubation at 37 ˚C, 20 min → 4 ˚C

***Polymerase chain reaction..***1. For the amplification of the expressed mRNA of **46 kDa** calreticulin, the following primer pair was used:

5’GAAACATGAGCAGAACATCGACTGTG,

and 3’CAAAGTTATCATAGGCATAGAGATACTGG.

These primers are suitable for the amplification of mRNA ranging from 356 to 1000 bp of the original sequence (McCauliffe 90). The amplified DNA was 644 bp long.

2. The primers used for the PCR of the **52 kDa** Ro were:

5’ TCTAGGATTCACGCAGAGTTTGTGC,

and 3’ ATCTCTCTTCATTTCCAGGTATGCTC.

The length of the amplified DNA was 448 bp, which corresponds to the sequence of Ro from 5216 to 973 bp (Chan 91).

3. The primers used for the amplification of the **60 kDa** mRNA were:

5’ AGTCATTTAGTCAAGAAGGCAGAACC,

and 3’ GACCTGTCTTGTAAGTTTCTAATGCG.

These were used to amplify a 771 bp long piece of the original mRNA (Ben-Chetrit 89) ranging from 471 to 1187 bp.

4. The primers used to amplify the mRNA of the **La exon 1** form were:

5’ CTTCTGTGGGCCGGAACCTTAAAG,

and 3’ CTGTTGTTAGACGGTTCAACCTGTTG,

where the 32 primer was also used to amplify the other La mRNA form. The amplified piece was from 31 to 190 bp (Tröster 94) resulting in a 221 bp long DNA.

5. The primers used for the amplification of the **La exon 1’** form were:

5’ TTCTAGTCTCACCGAAGGCTTGTG,

and the same 3’ primer was used as for the amplification of the exon 1 form. From the starting –237 bp, a 427 bp long DNA was amplified (Tröster 94).

6. To amplify **the exon 1”** mRNA of the La gene, the primers used were:

3’ TTTCAGTGTGAAACGGGAAAACGTG,

and the same 5’ primer was used as for exon 1 and 1” forms. The amplified DNA was 428 bp long.

The levels of G3PDH mRNAs were also measured as controls. The 983 bp product was amplified using a Clontech amplimer set.

Samples were amplified in a 25 µl final reaction volume: 1 µl cDNA, 2.5 µl 10x Perkin-Elmer Stoffel fragment buffer, 3 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP, 1 µl of each primers and 5 U AmpliTaq DNA Polymerase (Applied Biosystem).

All the amplifications comprised 40–45 cycles of denaturation at 94 °C for 15 s, annealing at 65–71 °C (depending on the primers) for 15 s, and synthesis at 72 °C for 30 s, and were performed in a Perkin-Elmer PCR 9600 thermocycler. The amplified DNAs were run in a 3% agarose gel (60 V, 10 min; 100 V 45 min). The gel was stained with ethidium bromide and the bands were visualized under UV light. Photographs were taken using a Polaroid instant camera.

# V. PATIENTS AND METHODS

## IL-10 polymorphism

***Patients and controls.*** Ninety-nine patients with Sjögren's syndrome were chosen from the patients of the 3rd Department of Medicine at the University of Debrecen, Hungary. All patients fulfill the European-American Consortium criteria for primary Ss (3 or more: xerophthalmia, xerostomia, immunologic features (Vitali 2002). At research the patients are aged between 25 and 75 years (mean 55.6 ± 11.8 years). Ninety-seven patients are female and two are male.

One hundred and thirty-five healthy controls aged between 23 and 78 years (mean 39.9 ± 10.9 years) were collected by advertisement through the University Medical School of Debrecen. Ninety-two controls are female and forty-three are male.

***DNA extraction..*** For IL-10 PCR-RFLP assay the genomic DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (QIAGEN)(Cat. No. 51106) according to the manufacturer's instructions. The concentrations of the DNAs were measured by spectrophotometer (Shimadzu UV-2100).

***PCR reaction..*** PCR for position -1082 was performed using the following primers 5' TCT GAA GAA GTC CTG ATG TCA CTG 3' and 5' ACT TTC ATC TTA CCT ATC CCT ACT TCC 3' (Lowe 2001). The reverse primer contained an additional Mnl I restriction site (underlined nucleotide) within the primer recognition sequence. Samples were amplified in a 25 µl final reaction volume: 250 ng of sample DNA, 1x Perkin-Elmer Stoffel fragment buffer, 3 mM $MgCl_2$, 0.4 mM dNTP, 0.11 nmol of each primers and 5 U AmpliTaq DNA Polymerase (Applied Biosystem). PCR amplifications were done in a Perkin-Elmer PCR 9600 thermocycler. Cycling conditions for -1082 amplifications were as follows: initial denaturing step of 94 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 67 °C for 30 sec, extension at 72°C for 30 sec and a final incubation at 72 °C for 5 minutes.

***RFLP.*** Restriction Fragment Length Polymorphism (RFLP) was performed using Mnl I restriction endonuclease (New England Biolabs). PCR products were digested with 1 U restriction endonuclease at 37 °C for 16 hours.

***Electrophoresis.*** The products were analyzed after electrophoresis (60 V, 10 min; 100 V 3 h) on 3 % agarose gel staining with SybrGreen (SIGMA). The bands were visualized under UV light by Multimage Light Cabinet AlphaImager 1220 Documentation Analysis System (Alpha Innotech Corporation). The positive control was a „common" 65 bp fragment that is generated upon digesting the genomic PCR product for all genotypes. The negative control

was the undigested PCR fragment (198 bp). Each of the genotyping reactions was valid, as the 65 bp product was observed in all reactions in the presence of the Mnl I digestion. In addition to the 65 bp fragment, in the presence of the G containing allele two additional were seen: one of 39 bp and one approximately 94 bp, the A containing allele yields a single additional product of approximately 133 bp (Fig. 5.).

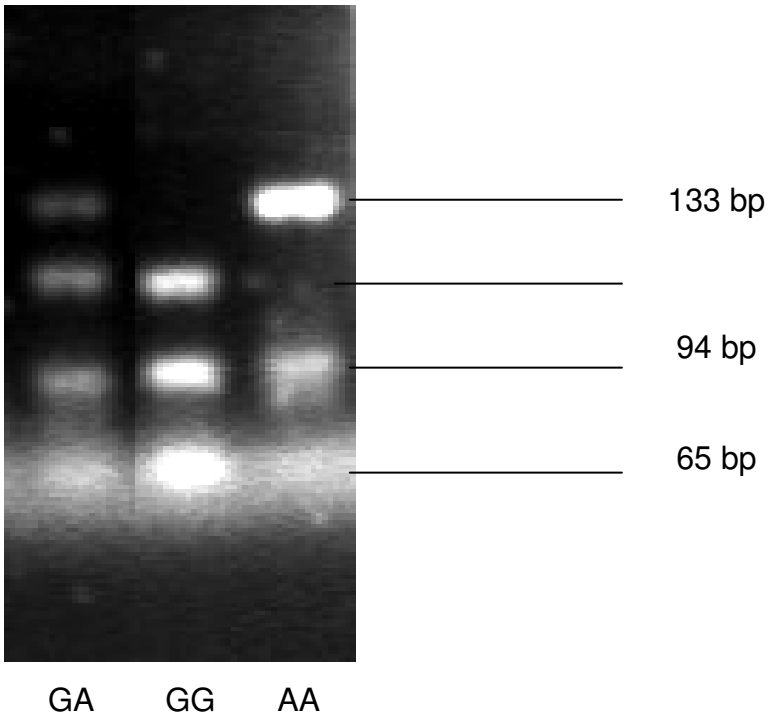



**Fig. 5.** The three genotypes on the agarose gel

***IL-10 levels.*** Serum IL-10 levels were measured in all patients and controls by classical, sandwich Enzyme-Linked ImmunoSorbent Assay (Human IL-10 BD OptEIA ELISA set, BD Biosciences Pharmingen)(Cat. No. 555157) method by following the manufacturer's instructions.

***Statistical analysis.*** Considering all the genotypes, the statistical power was 90%, therefore the applied number of individuals was appropriate (nQuery Advisor 5.0). Concentrations of IL-10 were compared by Kruskal-Wallis ANOVA analysis of variance and

Student-t test for two samples. Gene frequencies were calculated by gene counting and expressed as the ratio of the number of each allele observed within a locus to the total number observed in that locus. We used Chi-square test (www.fon.hum.uva.nl/Service/Statistics.html) to compare gene frequencies between Ss patients and controls.

# VI. RESULTS

## mRNA expression of Ro/SSA and La/SSB autoantigens in HaCaT cells

### 1. Detection of the basal level of the three Ro/SSA antigen mRNAs and that of the different splicing variants of the La/SSB mRNA in HaCaT cells.

Using the PCR technique we were able to detect the mRNA of all three forms of Ro/SSA protein antigens (46 kDa, 52 kDa and 60 kDa) in HaCaT cells for the first time (Fig. 6B). The basal level of the 60 kDa Ro/SSA protein mRNA was the lowest and the basal levels of the mRNA of the 46 kDa and 52 kDa proteins were nearly equal in all experiments. It has previously been demonstrated that in lymphocytes of patients with Sjögren's syndrome there are three different splicing variants of La/SSB mRNA (Fig. 1) and we were able to detect these splicing variants in HaCaT cells for the first time (Fig. 6A). The basal level of La exon 1' mRNA was much lower in HaCaT cells than in lymphocytes. Fig. 6A shows the approximate ratios of the three different forms.

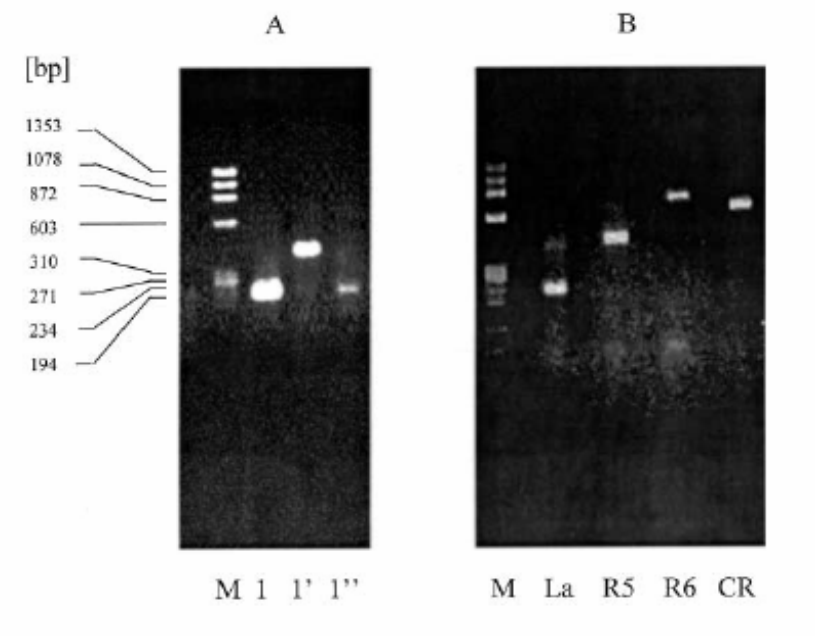
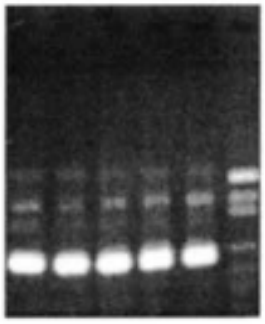



**Fig. 6.** Expression of the different mRNAs of La and Ro genes. A. Detection of the three different forms of La mRNA in HaCaT cells. The picture shows the approximate ratios of the expression of the three mRNAs. 1= exon 1, 1'= exon 1', 1''=exon 1'', M=marker B. La exon 1 and the three Ro mRNAs in HaCaT cells. La = La exon 1, R5= Ro 52 kDa, R6= Ro 60 kDa (marker:φX174)

## 2. Investigation of the effect of UVB irradiation on the expression of Ro/SSA and La/SSB mRNA forms in HaCaT cells

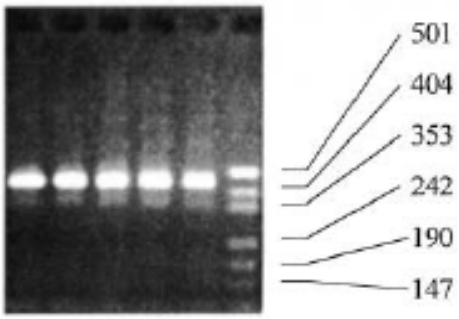
UVB irradiation at a dose of 200 J/m$^2$ did not result in any change in the mRNA levels of the 60 kDa and the 52 kDa Ro/SSA proteins after 12, 24, 48 and 72 h in HaCaT cells (Fig. 7). In contrast, a considerable increase in the mRNA level of the 46 kDa form, calreticulin, was detected after 24 and 48 h. Although there was a difference in the basal mRNA levels of the 24 and 48 h samples, the UV-irradiated cells always showed higher mRNA levels than any of the control cells. Concerning the mRNA levels of the splicing variants of the La/SSB protein, there was no significant change in either the La/SSB exon 1' level or the La/SSB exon 1 level as a function of time. No changes in the mRNA levels in the G3PDH controls were detected (Fig. 7E).

A



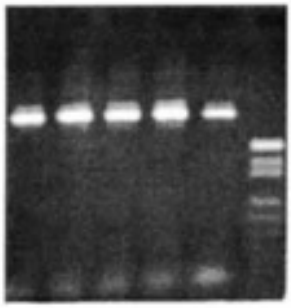
C2C4 U2U4 S M

B



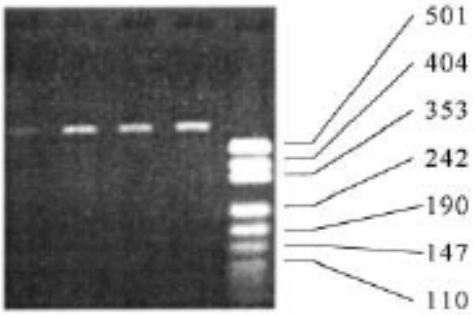

C2C4 U2U4 S M

C



C2C4 U2U4 S M

D




C2 C4 U2 U4 M

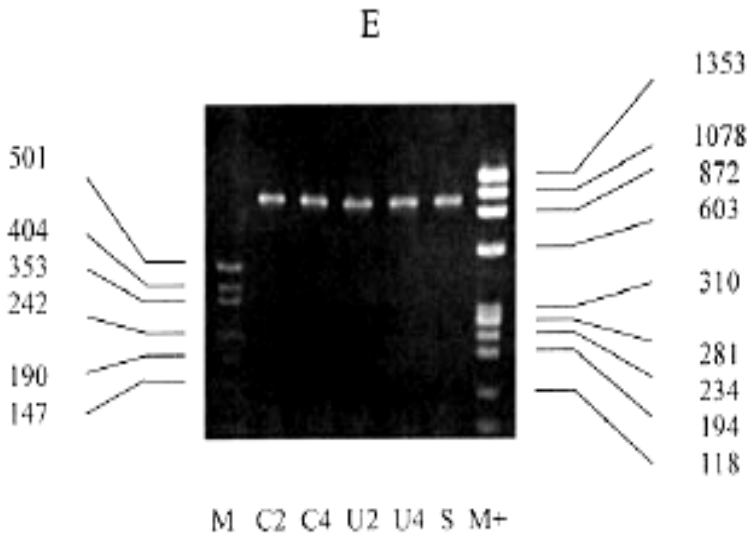
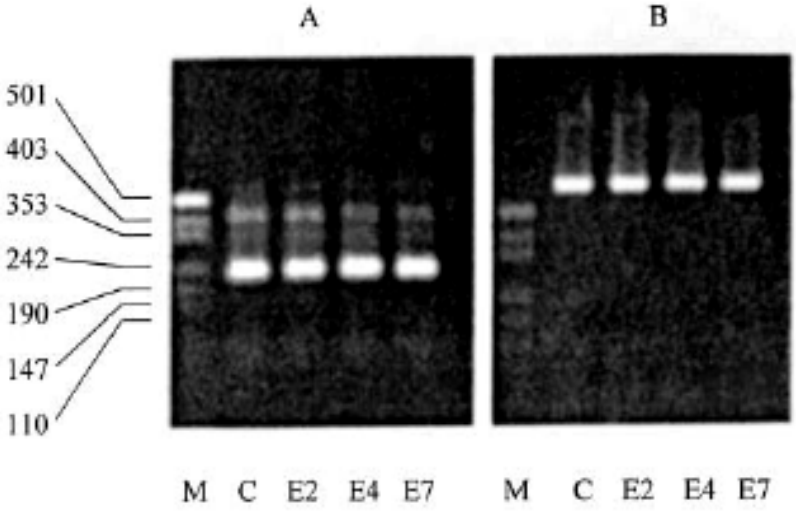
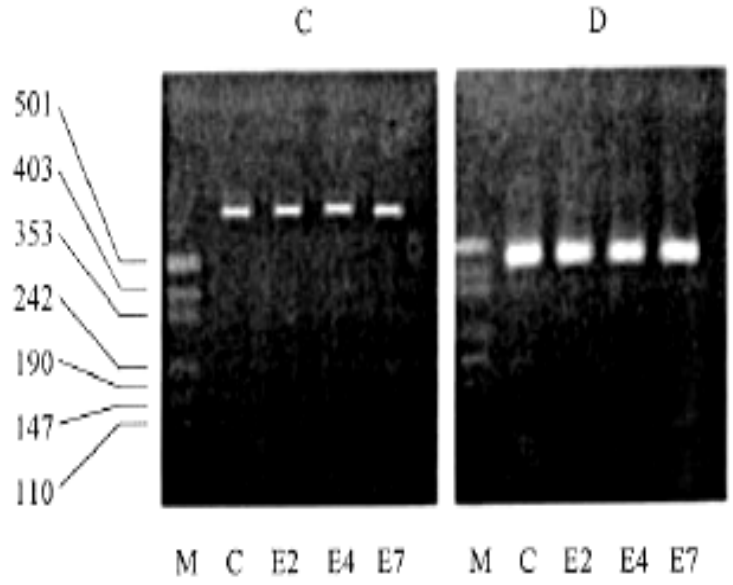



**Fig. 7.** Effect of UVB irradiation on the expression of La and Ro mRNAs in HaCaT cells. A. La exon 1 and exon 1' B. Ro 52 kDa C. Ro 60 kDa D. calreticulin E. G3PDH controls (C2=24 h, C4=48 h, U2=24 h after UVB, U4=48 h after UVB, S=unirradiated lymphocytes of SCLE patients, M=marker)

## 3. Investigation of the effect of 17-β-estradiol treatment on the expression of Ro/SSA and La/SSB mRNA forms in HaCaT cells

No changes were detected in the mRNA levels of the 46 kDa, 52 kDa and 60 kDa Ro/SSA proteins in HaCaT cells treated with 17-β-estradiol after 12, 24, 48 and 72 h (Fig. 8), and no change was detected in the levels of the control G3PDH mRNAs either. Similarly, no change was detected in the levels of La/SSB exon 1 mRNA, but a gradual decrease was found in the mRNA levels of La exon 1'. The change occurred as early as after 12 h and the mRNA expression had decreased to a very low level after 72 h.


A
B
501
403
353
242
190
147
110
M C E2 E4 E7
M C E2 E4 E7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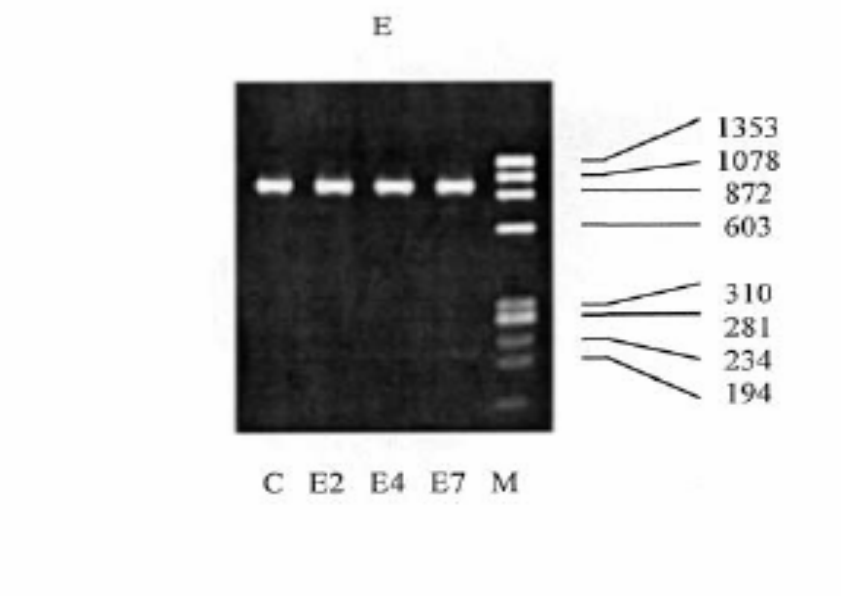


**Fig. 8.:** Effect of estradiol on the expression of La and Ro mRNAs in HaCaT cells. A. La B. calreticulin C. Ro 52 kDa E. Ro 60 kDa E. G3PDH controls (C=control, E2=after 24 h, E4= after 48 h, E7=after 72 h estradiol treatment, M=marker)

## IL-10 polymorphism

### 1. IL-10 plasma levels in Ss patients and controls

The IL-10 plasma levels were higher in the primary Ss patients (36.38 ± 57.52 pg/ml n=99) compared with the healthy controls (9.89 ± 20.28 pg/ml, n=135), and this difference was significant ($p=10^{-6}$) (Table 1) (Fig. 9). The patients were then classified according to their genotypes, and effects of these on the plasma IL-10 levels were analyzed (Fig. 10). In the control group, after classification by genotypes, we found higher IL-10 levels among the healthy controls who were carriers of the GG genotype (17.68 ± 23.2 pg/ml) than in the other two carriers (AA 8.98 ± 16.45 and GA 8.55 ± 21.05 pg/ml). This difference was also significant ($p<0.01$) (Fig. 10).

| *IL-10 levels mean ±* | *Sjögren's syndrome pg/ml (n)* | *Control pg/ml (n)* | *p* |
|---|---|---|---|
| **all** | 36.38 ± 57.52 (99) | 9.89 ± 20.28 (135) | $10^{-6}$ |
| **GG** | 37.62 ± 59.32 (13) | 17.68 ± 23.20 (18) | 0.202 |
| **AA** | 46.38 ± 59.39 (33) | 8.98 ± 16.45 (38) | 0.0003 |
| **GA** | 29.86 ± 56.09 (53) | 8.55 ± 21.05 (79) | 0.0028 |

**Table 1.** IL-10 levels in pg/ml in Ss patients and controls.

## 2. Polymorphism of the IL-10 nt -1082 promoter in the Ss patients and controls

After determining the single nucleotide polymorphism of nt -1082 for each subject, the genotype distribution, genotype and allele frequencies in Ss patients and healthy controls were analyzed. The results are summarized in Table 2. The frequency of the IL-10 GG genotype was the same in the Ss patients and controls (0.131 vs 0.134). The AA genotype frequency was almost the same in the patient group compared with healthy controls (0.333 vs 0.281). The frequency of the heterozygous genotype was similar in the Ss patients (0.535) and in control subjects (0.585).

| *Genotype* | *Sjögren's syndrome % (n)* | *Control % (n)* | $X^2$ | *p* |
|---|---|---|---|---|
| **GG** | 0.131 (13) | 0.134 (18) | 0.02 | 0.887 |
| **AA** | 0.333 (33) | 0.281 (38) | 0.50 | 0.479 |
| **GA** | 0.535 (53) | 0.585 (79) | 0.39 | 0.532 |
| ***Allele*** | | | | |
| **G** | 0.399 | 0.426 | - | - |
| **A** | 0.601 | 0.574 | - | - |

**Table 2**. IL-10 genotype frequencies in Ss patients and controls.

## 3. Polymorphism of the IL-10 nt -1082 promoter and Ss features

We summarized the main epidemiologic, some clinical and immunologic Ss features according to genotype in Table 3. We did not observe a significant difference in the onset of the disease. Systemic involvement was more frequent in GG genotype carriers, although the difference did not reach statistical significance (0.846 vs 0.154). In the other two genotype groups there were not any differences related to the presence or absence of systemic involvement. No significant differences in the haematologic (antinuclear antibodies, rheumatoid factor, SSA, SSB antibodies) parameters were observed in carriers of the three genotypes.

| | ***GG*** | ***AA*** | ***GA*** | ***Total*** |
|---|---|---|---|---|
| **Sex (Female/Male)** | 13/0 | 31/2 | 53/0 | 97/2 |
| **Age onset (year)** | 49.38±8.05 | 49.03±13.6 | 47.45±10.95 | 48.23±11.52 |
| **Systemic involvement % (n)** | 84.62% (11) | 51.52% (17) | 56.6% (30) | 58.59% (58) |
| **SSA/B % (n)** | 46.15% (6) | 54.55% (18) | 58.49% (31) | 31.31% (55) |
| **Antinuclear antibodies % (n)** | 30.77% (4) | 24.24% (8) | 24.53% (13) | 25.25% (25) |

**Table 3.** Frequency of the main epidemiologic, clinical and immunologic Ss features according to the genotype.

## 4. IL-10 plasma levels and Ss features

Increased plasma IL-10 levels were found in SSA and SSB antibody positive (27.71 ± 35.46 pg/ml) and negative (41.59 ± 70.92 pg/ml) primary Ss patients compared with healthy controls (9.89 ± 20.28 pg/ml) ($p = 5.13x10^{-5}$ and $p = 3.21x10^{-6}$), although between the two pSs groups, SSA/B positive and negative, there was no significant difference ($p = 0.254$). Hypergammaglobulinemia is a characteristic of the Ss; the high IL-10 level may have a role in

the development of it. In patients with raised IgG levels (>16g/l), we found significantly higher IL-10 levels compared with control subjects (48.59 ± 63.36 pg/ml versus 9.89 ± 20.28 pg/ml, $p = 6.7x10^{-8}$), but there was a significant difference between patients with normal IgG levels and the controls (31.9 ± 56.89 pg/ml versus 9.89 ± 20.28 pg/ml, $p = 5.62x10^{-5}$). The patients were then classified according to their autoantibody positivity, and the effects of these on the plasma IL-10 levels were analyzed. In all the patients who were negative for SSA/B, the levels of IgG were normal (7-16 g/l), but the IL-10 levels were increased compared with healthy controls (42.38 ± 71.36 pg/ml versus 9.89 ± 20.28 pg/ml, $p =2.2x10^{-6}$). In almost half of the patients, who were positive, a raised IgG level was found with significantly higher IL-10 levels than in controls (37.76 ± 47.27 pg/ml versus 9.89 ± 20.28 pg/ml, $p=1.52x10^{-5}$). In the other half of the SSA/B positive pSs patients with normal IgG levels, the IL-10 levels were higher as well (20.58 ± 22.45 pg/ml versus 9.89 ± 20.28 pg/ml, p=0.016).

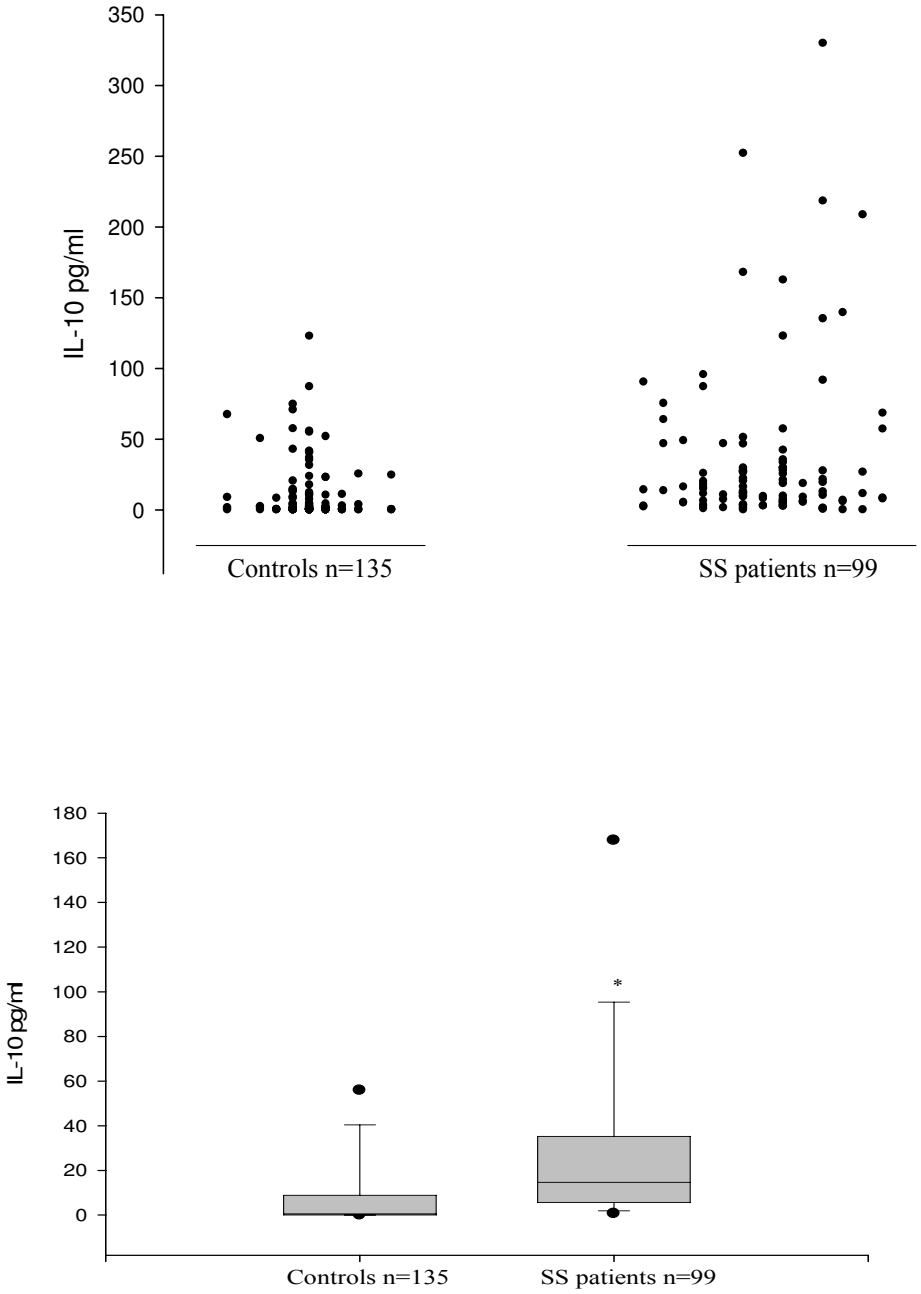


**Fig. 9.:** Plasma interleukin-10 concentration in healthy subjects and Ss patients. Each symbol in panel A represents one sample. Columns represent the median and $5^{th}/95^{th}$ percantiles. * $p<0.05$ between the controls and Ss patients.

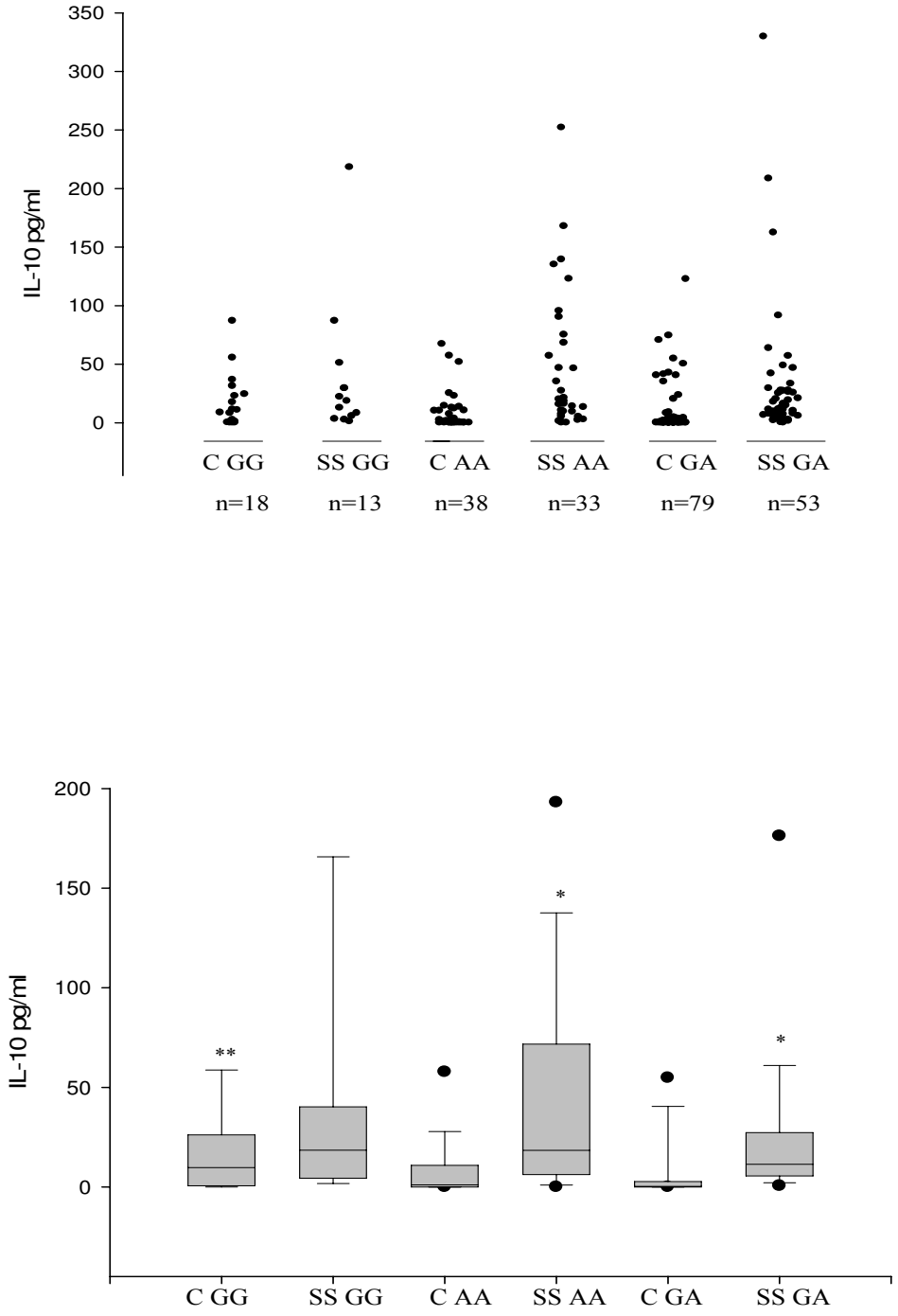


**Fig. 10.** Effects of IL-10 genotypes on the plasma IL-10 concentration in healthy subjects and Sjögren's syndrome patients (C=control, Ss=Sjögren's syndrome). Each symbol in panel A represents one sample. Columns represent the median and $5^{th}$/$95^{th}$ percantiles. * $p<0.05$ between the controls and Ss patients. ** $p=0.01$ within the control group (GG and AA, GA).

# VII. DISCUSSION

## mRNA expression of Ro/SSA and La/SSB autoantigens in HaCaT cells

Epidermal keratinocytes, especially the basal cells, are the targets of immunological damage in photosensitive lupus, in SCLE and in NLE. HaCaT cells are spontaneously transformed human keratinocytes that show some characteristics of basal epidermal keratinocytes (Boukamp 88). Our results demonstrate that in HaCaT cells, as in cultured human keratinocytes, one can find the mRNA forms of the 46 kDa, 52 kDa and 60 kDa Ro/SSA protein antigens. Kawashima et al. have also found the three Ro/SSA polypeptides in another transformed human keratinocyte cell line, A431 (Kawashima 94). They found that the basal level of the 60 kDa polypeptide was the lowest, and that the calreticulin was the highest. In HaCaT cells, we also found that the mRNA level of 60 kDa form was lower than the levels of calreticulin and the 52 kDa form.

In peripherial blood lymphocytes from patient with primary Sjögren's syndrome, Tröster et al. (Tröster 94) identified two alternative types of La/SSB mRNA. These were the results of promoter switching combined with an alternative splicing mechanism. In HaCaT cells we also found the two alternative mRNA types of La/SSB (La exon 1' and exon 1'') as well the common variant (La exon 1). The mRNA level of La exon 1' was much lower in HaCaT cells than in lymphocytes of patients with Sjögren's syndrome.

Originally LeFeber et al (LeFeber 84), and later other investigators (Furukawa 88, 90 Jones 92), suggested that Ro/SSA antigens and La/SSB antigens, normally present in the nucleus and cytoplasm of human keratinocytes could be induced to appear on the cell surface following exposure to UV radiation. UV irradiation of cultured keratinocytes induces

apoptotic changes and the clustering of autoantigens at the cell surface with smaller blebs containing the Ro antigen, calreticulin, ribosomes and endoplasmic reticulum and larger blebs containing Ro, La and nucleosomal DNA (Casciola-Rosen 97). However, there may be another way for Ro and La proteins to appear on the cell surface. Physiological UV doses, especially UVB, result in the production by epidermal keratinocytes of a number of molecules that can influence cutaneous inflammation without apoptotic changes (Bennion 97). Some of these molecules may be directly induced by UV radiation after activating transcription factors by singlet oxygen-dependent mechanisms (Grether-Beck 96). It is possible that Ro and La proteins appear on the cell surface following exposure to UV radiation as a result of induced transcriptional activation of their regulatory transcription factors. It has been suggested but not yet proven, that genetic polymorphism within the regulatory pathways for these molecules could be a predisposing factor for photosensitive cutaneous lupus erythematosus (Werth 97).

When keratinocytes are exposed to UVB radiation, it is not clear whether all genes encoding Ro/SSA proteins or only a limited subset of this complex are upregulated. Therefore, we examined the expression of the mRNA of the three Ro/SSA polypeptides that have been cloned and sequenced (60, 52 and 46 kDa) after UVB irradiation. After a physiologically relevant dose of UVB radiation, a significantly higher level of mRNA of the calreticulin was observed in HaCaT cells after 24 h, while no changes in the mRNA levels of the 52 and 60 kDa Ro/SSA were found. Although there was a difference in the mRNA levels in the 24 h and 48 h control samples, the UV-irradiated cells had higher levels than any of the control samples. The variation in the basal mRNA levels of calreticulin can not easily be explained since these levels can be induced by several different factors such as cell stress (e.g. heat shock), perturbation in the normal endoplasmic reticulum, heavy metals etc. (Eggleton 99).

In accordance with our results, Kawashima et al. have reported that only calreticulin, and not the 60 and 52 kDa polypeptides, is upregulated in total cellular and cell surface expression in A431 cells after UVB irradiation (Kawashima 94). Autoantibodies against the Ro/SSA and La/SSB antigens are frequently produced by patients with particular autoimmune disease. Several lines of evidence (McCauliffe 89, Sontheimer 90) indicate that these autoantibodies play not only diagnostic, but also pathogenic role in these diseases (especially in SCLE and NLE). The strongest evidence comes from observations of patients with NLE. In the NLE syndrome the development of skin disease corresponds to the presence of maternal IgG antiRo/SSA antibodies in the neonate, and the development of heart block is highly associated with antibodies to the same antigen (Lee 84, Buyon 98). The pattern of cutaneous IgG deposition in SCLE has been reproduced by the infusion of purified antiRo/SSA autoantibodies into human skin-grafted mice (Lee 89). AntiLa/SSB antibodies may also be involved because antibodies against this antigen are usually detectable in antiRo/SSA-positive serum and there are patients with SCLE with high antiLa/SSB titers but with very low titers of antiRo/SSA antibodies (Sontheimer 82, Harley 84).

The display of Ro/SSA and La/SSB autoantigens on the surface of normal human keratinocytes following UVB irradiation and the resulting binding of the circulating antiRo/SSA and antiLa/SSB autoantibodies to their antigens could result in tissue injury through complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity (Kawashima 94). Our results correlate with those of Kawashima et al. and calreticulin could be a critical component of this Ro/SSA complex that appears on the cell surface of keratinocytes after UVB irradiation.

The hormonal milieu may also play an important role in Ro/SSA and La/SSB associated disease. A female predominance in SCLE, SLE, NLE and Ss is well documented. The disease activity increases during menses and during use of oral contraceptives. Moreover,

androgen treatment delays the onset and severity of autoimmune phenomena in female NZB/NZW F1 mice (Wang 96, Rose 44, Melez 80, Roubinian 78). Estrogen treatment of cultured keratinocytes results in the increased expression of Ro/SSA and La/SSB antigens on the cell surface (Lehmann 97). Our results from the 17-beta-estradiol stimulation differ from those of Wang and Chan (Wang 96) who found that at concentrations of $10^{-8}$ to $10^{-7}$ M, 17-β-estradiol induces both 52 kDa and 60 kDa Ro/SSA mRNAs in human keratinocytes by up to fivefold compared with untreated cells. In HaCaT cells 17-beta-estradiol at a concentration of $10^{-8}$ M, did not cause significant changes in the levels of mRNA of calreticulin or that of the 60 kDa and 52 kDa Ro/SSA proteins. It is difficult to understand why HaCaT cells show a different behavior compared to cultured human keratinocytes under estrogen stimulation, while after UVB irradiation they react similarly. However, this duality in HaCaT cells has also been observed by Lehman who demonstrated that HaCaT cells can be used in studies of the vitamin D3 pathway and its relationship to proliferation. Differences in calcitriol synthesis and catabolism from those of cultured primary keratinocytes, however, must be considered (Lehmann 97).

Concerning the different mRNA forms of the La/SSB protein, we found no change in the levels of La exon 1' mRNA after UVB irradiation. Estrogen stimulation, however, resulted in a decrease in the exon 1' mRNA levels, but the significance of this finding is not yet clear. However, Grölz et al. (Grölz 98) have reported that NF-kappaB element is present in the regulatory region of exon 1' variant and in turn could play a role in the regulation of this mRNA. This NF-kappaB element could be influenced by active oxygen species produced as a result of, for example, UV irradiation, and this element could also be controlled by different hormones. Other recent results (Carter 2000), emphasizing the importance of this topic, also show differences in the expression of the two La mRNAs as a function of chemicals and mitogens.

In conclusion, we detected the mRNA forms of all three different Ro/SSA antigens and that of three La/SSB species produced by alternative splicing in transformed human keratinocytes. After UVB irradiation the mRNA levels of calreticulin increased and treatment of the cells with 17-beta-estradiol resulted in a gradual decrease in the levels of La exon 1' mRNA. Our results strengthen the belief that Ro and La antigens participate in the pathogenesis of different autoimmune disease, but further studies are required to elucidate the exact pathomechanism involved.

## IL-10 polymorphism

Differences of more than 3 million nucleotides can bee seen comparing the genomes of two members of the same population as a result of single nucleotide polymorphism (SNP) (Shastry 2002). There are other types of genetic differences (e.g. other types of polymorphisms, insertions, deletions, duplications, etc.) as well, but one of the more important genetic alterations is the SNP (Vignal 2002).

SNPs can be found in different parts of the genome, such as in coding or non-coding regions, with or without any effects on the gene product. Sometimes an SNP in the non-coding region (e.g. in a promoter) influences the transcription of the gene, but in other instances even an SNP in the coding exon has no effect on the protein structure and/or function. Synonym SNPs do not change the encoded amino acids, but non-synonym SNPs induce new ones. The resulting altered proteins could possess different features comparing to the non-altered, common forms. In the cases of altered proteins the difference could be seen e.g. in conformational alteration that eventually could lead to a change in enzyme activities, in

certain cases even the entire function of the whole protein could be diminished. Therefore, individuals with different SNPs could have slightly different metabolic pathways.

After having sequenced the entire human genome there is a possibility to determine the individual differences (Cargill 99, Altshuler 2000). Some of the different approaches concerning the SNPs are: a whole chromosome (Mullikin 2000), a part of the genome (Haga 2002), and a complex disease (Emahazion 2001), or a single gene (Trikka 2002).

Knowledge on the genetic variability could lead to the resolutions of different biological problems. Evolutionary alterations and the trait of selection can be followed (Schmidt 2000, Akey 2002), among others, since most of the changes occur in highly recombinant regions (Lercher 2002): the evolution of species and that of interspecies can be traced. SNPs have another important role, for example, in pharmacology. It is well known that individuals tolerate drugs and environmental effects differently (Bell 2002, Mrash 2002). There are people with strong adverse reactions to certain medicaments: more than 100,000 people die yearly in the USA because of anaphylactic reactions (Pirmohamed 2001), nevertheless the knowledge on SNPs in metabolic genes could result even in a better formulation of medicaments too.

One of the well known parts of the SNP-theme is the disease susceptibilities. Series of publications support the idea that the presence of certain SNPs is in connection with the emergence of a disease since individuals with defined SNP suffer from a disease more frequently if one compare them to the healthy population. Only a few instances in a row: Alzheimer disease (Emahazion 2001), sickle cell anemia (Waterfall 2001), hyperlipidemia (Osawa 2001), schizophrenia (Li 2000), different malignancies (Trikka 2002), etc. Obviously not only single gene disorders can play a role in the pathogenesis of different diseases but several other factors as well (multiple genes, environmental factors, etc.).

A genetic contribution to the etiology of Ss is evidenced by the increased frequency of Ss in relatives and siblings of primary Ss patients. Multiple studies investigated the possible associations between IL-10 promoter polymorphisms in patients with SLE (Eskdale 97, Mok 98, Lazarus 97, Alarcon-Riquelme 99) or rheumatoid arthritis (Eskdale 98, Coakley 98). Studies have shown high levels of IL-10 in peripheral blood mononuclear cells and salivary glands in patients with primary Ss (Llorente 94), and other recent studies have analyzed the IL-10 promoter polymorphisms in patients with primary Ss (Rischmueller 2000, Hulkkonen 2001, Font 2002, Lester 2002, Origuchi 2003). An increased frequency of the GCC haplotype and a decreased frequency of the ACC haplotype were observed in Ss patients (Hulkkonen 2001, Font 2002) but there was no correlation between extraglandular manifestations and IL-10 haplotypes and no significant differences were found in clinical and immunologic features of Ss patients (Rischmueller 2000, Hulkkonen 2001, Font 2002), while Rischmueller et al. (Rischmueller 2000) and Lester et al. (Lester 2002) found no differences in the frequency of the IL-10 haplotypes.

Some recent studies indicate that SNPs can be rendered to individuals living a longer life than the average. Perhaps these results will not directly lead to the lengthening of the maximal life span; however, genes that play an important role in the aging process could be identified. In this respect SNPs are important factors in determining the information level of the cells of individuals, which determines the maximal life span (Semsei 2000), in turn SNP is one of the factors that determine the aging process. The frequency of diseases increases with age, so Ss, similarly to different malignant diseases (Cutler 1989), could have common roots with senescence (Urbán 2001). A polymorphism, the -1082G/A, in the promoter region of the gene was extensively studied with respect to the Ss and to aging as well. These results indicate that the -1082G haplotype is significantly higher in old men than in young controls while the number of the GCC/ATA genotype is significantly higher in women suffering from

Ss (Lio 2002, 2003b). Sjögren's syndrome is basically a women's disease with a higher than 90% involvement of women. According to our own results the AA homozygote type in the -1082 position is more elevated in Ss than in the age-matched controls, whereas the GG homozygote women are underrepresented compared to the AA homozygote women with Ss (although in the control group the frequency of the GG and AA types are similar). It is obvious that the extremely high age and a disease susceptibility are usually not determined by a single SNP, although the -1082G allele can be rendered to the possibility of high age (in men) and the -1082A one to the elevated Ss disease susceptibility (in women) (Bessenyei 2004).

In this study we analyzed the -1082 polymorphism of the IL-10 gene promoter and found that the frequency of the GG genotype was similar and the frequency of the AA genotype was increased to a lesser degree in primary Ss patients compared with the control subjects. These results are in agreement with a Japanese study where the GCC haplotype, which is predominant in white subjects, was less common in Japanese Ss patients, while the frequency of the AA genotype was higher (Font 2002). Moreover, we measured the levels of IL-10 in plasma and found that the presence of the disease is associated with high plasma levels of IL-10, which is in agreement with the results observed by Turner et al in mononuclear cell cultures (Turner 97) and with results presented by Llorente et al., in which elevated levels of IL-10 mRNA and IL-10 protein were found in peripheral blood mononuclear cell cultures in primary Ss (Llorente 94). Although results of Hulkkonen et al. (Hulkkonen 2001) showed higher IL-10 protein production in subjects who were negative for A at position -1082 compared to A positive individuals, we found that the plasma IL-10 levels were elevated considerably in those who carry the A allele at position -1082 compared to those who were negative for A allele nt-1082. The level of IL-10 is an important factor, because IL-10 and IL-6 play central roles in the maturation of plasma cells and activation of

immunoglobulin synthesis. We did not find a direct correlation between IL-10 levels and IgG levels or presence of SSA/B antibodies, but it is possible that the increased levels of IL-10 contribute to the development of hypergammaglobulinemia, which is characteristic of Ss, and promotes autoantibody production associated with primary Ss. Moreover patients with Ss have an increased risk of developing lymphomas, and it is possible that persistently high levels of IL-10 may contribute to this conditions as well (Levy 94).

Although a recent study found that the appearance of the disease was earlier in Ss patients carrying the IL-10 GCC haplotype (Font 2002), we could not find this kind of difference when we compared the age at onset of the three genotypes. Nevertheless, differences in the clinical and immunologic Ss features were observed in our patients with respect to the three different IL-10 promoter polymorphisms. We found an increased frequency of systemic involvement in GG genotype carriers, however, we found no correlation between immunologic (antiRo/SSA, antiLa/SSB) features and IL-10 genotypes. Thus, the IL-10 promoter polymorphism at nt-1082 seems to have some measurable influence on the clinical expression of primary Ss.

In conclusion we describe a high level of plasma IL-10 in patients with Sjögren's syndrome compared with healthy controls, and show that the -1082 GG genotype is not responsible for this phenotype. We found an increased frequency of the A allele and AA genotype in Ss patients similarly to the results of a Japanese study. IL-10 polymorphism may be an important component of the genetic background, the susceptibility, and clinical expression of primary Ss patients, but more extensive genetic tests should be done including the examination of other IL-10 polymorphisms.

# VIII. SUMMARY

Autoimmune diseases encompass a very complex clinical picture. Several factors may play a role in the onset of the autoimmune disease and the disease susceptibility such as genetic polymorphisms (e.g. interleukins), autoantibodies (antiRo/SSA, antiLa/SSB, etc.) and aging may play a role in it as well, since the onset of several autoimmune diseases is age-dependent.

Antibodies produced against the Ro/SSA and La/SSB autoantigens are not only of diagnostic value but they may even play a role in the pathogenesis of several autoimmune diseases (Sjögren's syndrome, subacute cutaneous lupus erythematosus, neonatal lupus erythematosus and systemic lupus erythematosus). Among other factors, ultraviolet (UV) radiation and also the hormonal milieu are well-known cofactors in the pathogenesis of these autoimmune diseases. The goal of our research was to study the possible alterations in mRNA levels of three different Ro antigens and that of two La species produced by alternative splicing in transformed human keratinocytes (HaCaT cells) after UVB irradiation as well as after 17-β-estradiol treatment. The polymerase chain reaction technique was used to determine the mRNA levels of the Ro and La species after 24, 48, and 72 hours of irradiation. mRNA levels of calreticulin increased as a function of time after UV irradiation but mRNA levels of Ro 52 kDa and 60 kDa Ro mRNAs were unaltered. After treating the cells with 17-β-estradiol, no change was observed in the levels of Ro mRNAs or La exon1 mRNA, but a gradual decrease was noted in the mRNA levels of La exon1'. The importance of alterations in the ratio of La exon1 to exon1' is supported by the observations in patients with Sjögren's syndrome, and our results strengthen their role in the pathogenesis of different autoimmune diseases.

A further aim of our study was to investigate the frequency of the -1082 polymorphism of the interleukin-10 (IL-10) gene and soluble IL-10 levels in Hungarian primary Sjögren's syndrome (Ss) patients. Ninety-nine Ss patients and 135 healthy volunteers were examined. Samples were analyzed by the PCR-RFLP method and IL-10 plasma levels were assessed by a commercial ELISA assay. IL-10 plasma levels were higher in the primary Ss patients (36.4 ± 57.5 pg/ml n=99) compared with healthy subjects (9.9 ± 20.3 pg/ml, n=135, $p=10^{-6}$). The elevated IL-10 phenotype of Ss patients was not associated with increased G allele frequency as reported earlier, while in the control group, we found higher IL-10 levels among the subjects who were carriers of the GG genotype (17.7 ± 23.2 pg/ml) as compared to the other two genotype carriers (AA 8.98 ± 16.5 and GA 8.5 ± 21.1 pg/ml, p=0.01). Our data do not support some previous observations that indicated an association between deregulated IL-10 secretion in Ss and higher G allele frequency. However, our results clearly demonstrate that GG homozygosity is associated with elevated IL-10 levels in apparently healthy subjects, but this can not account for the IL-10 related specific disease features observed in Ss. Thus, other genetic factors contribute to the clinical spectrum of this heterogeneous disease, at least in the Hungarian population.

# IX. ÖSSZEFOGLALÁS

Az autoimmun betegségek igen összetett kórképek, számos tényező befolyásolja a kialakulásukat: különböző genetikai eltérések, például az SNP-k (pl: IL-10), a különböző autoantitestek (többek között: antiRo/SSA, antiLa/SS), s az öregedés is szerepet játszhat, hiszen sok autoimmun betegség megjelenése életkorhoz köthető.

A Ro/SSA és La/SSB autoantigének ellen termelődő antitesteknek nemcsak a diagnosztikában, hanem az autoimmun betegségek patomechanizmusában is szerepük van. Több más tényező mellett az UVB besugárzás és a hormonális hatások is ismert kofaktorai ezen betegségeknek. A célunk az volt, hogy tanulmányozzuk a háromféle Ro/SSA és a kétféle La/SSB alternatív splicing formáinak különböző mRNS szintjeit transzformált humán keratinocitákban UVB besugárzás és 17-béta-ösztradiol kezelés hatására. PCR technika segítségével határoztuk meg a különböző mRNS szinteket 24, 48, 72 órás UVB besugárzás után. A kalretikulin mRNS szintje megnövekedett, míg az 52 és 60 kDa Ro/SSA mRNS–ek szintje nem változott. Ösztradiol kezelés hatására nem volt változás a Ro/SSA és az La/SSB exon1 mRNS szintekben, de csökkent az La/SSB exon1’ mRNS szint. A két variáns (exon1 és exon1’) arányának szerepe már bizonyított Sjögren szindrómában, s a mi eredményeink is megerősíik azt a feltételezést, miszerint a Ro és La antigének részt vesznek a különböző autoimmun betegségek patogenezisében, így a Sjögren szindrómáében is.

Az IL-10 promóterének -1082-es pozíciójában levő polimorfizmust és a szolubilis IL-10 szinteket vizsgáltuk Sjögren szindrómás betegek és egészségesek csoportjában. PCR-RFLP módszert alkalmaztunk a genotípusok meghatározásához, a szérum IL-10 szintjét ELISA módszerrel mértük. Az IL-10 szignifikánsan magasabb volt a Ss betegek (36,4 ± 57,5 pg/ml) esetén, mint a kontroll személyekben (9,9 ± 20,3 pg/ml; $p=10^{-6}$). A magas citokintermelő képességgel jellemezhető fenotípus és a nagyobb G allél gyakoriság között

nem találtunk összefüggést a Ss betegekben szemben a korábbi vizsgálatok eredményeivel, viszont a kontroll csoportban emelkedett IL-10 szinteket detektáltunk azok között, akik GG genotípus hordozók voltak (17,7 ± 23,2 pg/ml), összehasonlítva a másik két genotípust hordozó csoporttal (AA: 8,98 ± 16,5 pg/ml; GA: 8,5 ± 21,1 pg/ml; p=0,01). Vizsgálataink nem támasztják alá a korábbi vizsgálatokat, miszerint Ss-ben összefüggés lenne a magas G allél gyakoriság és a szabályozatlan IL-10 szekréció között. Az eredményeink szerint viszont a GG homozigótaság kapcsolatban van az emelkedett IL-10 szinttel az egészséges populációban, de ez nem magyarázza az IL-10-zel összefüggésbe hozható azon specifikus betegség paramétereket, melyeket általában vizsgálnak Ss-ben. Valószínűleg más genetikai faktorok is részt vesznek a betegség klinikai spektrumának kialakításában a magyar beteg populációban.

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# XII. APPENDIX

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