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Cutaneous lupus erythematosus and immunoreactivity in patients with Ro/SSA autoantibodies

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To my Family

ABSTRACT

Lupus erythematosus (LE) is a heterogenous autoimmune disorder with many different clinical presentations. Cutaneous lupus erythematosus (CLE) is subdivided into acute, subacute and chronic CLE. Population-based epidemiologic studies of CLE are lacking and the pathogenesis is not fully understood. A major target for the autoimmune response in lupus is the Ro52 antigen, and Ro/SSA autoantibodies are frequently found in sera from patients with lupus. The focus of this thesis has been to estimate the incidence and prevalence of subacute CLE (SCLE) in Stockholm, Sweden, to characterize Ro/SSA-positive patients, both clinically and serologically, and to study the skin as a target organ to learn more about the autoimmune inflammatory process.

By studying serology-based registers of Ro/SSA autoantibody positive patients and by the use of questionnaires, we could estimate the incidence of Ro/SSA-positive SCLE to 0.7 per 100,000 persons per year and the prevalence to 6.2-14 per 100,000 persons. Self-reported photosensitivity was found in 54% of the Ro/SSA-positive patients and polymorphous light eruption was found to be more common than in the general population. Smoking was also more common. Serologic analysis using enzyme linked immunosorbent assay revealed higher levels of autoantibodies binding Ro52 and La antigens in CLE patients with systemic manifestations compared to those with the disease confined to the skin, indicating different pathogenetic mechanisms for different clinical presentations. The fine specificity of Ro/SSA could thus serve as a tool to reveal predisposed patients at risk of developing systemic disease. Furthermore, a prospective study of our cohort revealed a dynamic disease process in Ro/SSA-positive patients in a short perspective of two years, with the development of either a new or an additional autoimmune disease as well as drug induced SCLE.

Immunohistochemical studies of skin biopsies revealed increased cytoplasmic and extracellular expression of the proinflammatory cytokine high mobility group box chromosomal protein 1 (HMGB1) in both spontaneously occurring as well as in photoprovoked lesions of CLE compared to normal controls. These findings imply that HMGB1 is involved in the inflammatory autoimmune process of CLE and may thus constitute a potential therapeutic target. We were also able to demonstrate TNFα and IL-1β expression in CLE lesions which together with HMGB1 can result in sustained inflammation in CLE. The Ro52 antigen was studied and found to be overexpressed in systemic LE (SLE) and Sjögren's syndrome patients and to function as an E3 ligase regulating proliferation and apoptosis. Ro52 may thus hypothetically contribute to the autoantigenic load and induction of autoimmune B and T cell responses observed in Ro/SSA-positive CLE patients as well.

In conclusion, SCLE is a rare subset of lupus but our data together with prevalence estimations for other subsets, show that CLE is probably a more prevalent disease than SLE. Guidelines of care based on our findings will include councelling concerning smoking cessation, sun-protection and avoidance of photosensitizing drugs. Our findings also demonstrate the importance of awareness of the manifestations Ro/SSA-positive patients are at risk of developing, and underline the need for regular clinical follow-up for these patients.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **A serology-based approach combined with clinical examination of 125 Ro/SSA-positive patients to define incidence and prevalence of subacute cutaneous lupus erythematosus**
K Popovic, F Nyberg, M Wahren-Herlenius, F Nyberg
Arthritis Rheum 2007; 56: 255-264.

II. **Fine specificity of the Ro/SSA autoantibody response in relation to serological and clinical findings in 96 patients with self-reported cutaneous symptoms induced by the sun**
K Popovic, S Brauner, M Ek, M Wahren-Herlenius, F Nyberg
Lupus. Accepted for publication.

III. **A prospective study of 102 anti-Ro/SSA positive patients: Two-year follow-up with clinical examination and questionnaires**
K Popovic, M Wahren-Herlenius, F Nyberg
Submitted for publication.

IV. **Increased expression of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in skin lesions of patients with lupus erythematosus**
K Popovic, M Ek, A Espinosa, L Padyukov, H Erlandsson Harris, M Wahren-Herlenius, F Nyberg
Arthritis Rheum 2005; 52: 3639-3645.

V. **Extracellular and cytoplasmic translocatiom of HMGB1 coincides with the peak of clinical activity in experimentally UV-induced lesions of cutaneous lupus erythematosus**
V Barkauskaite, M Ek, K Popovic, H Erlandsson Harris, M Wahren-Herlenius, F Nyberg
Submitted for publication.

VI. **The Sjögren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death**
A Espinosa, W Zhou, M Ek, M Hedlund, S Brauner, K Popovic, L Horvath, T Wallerskog, M Oukka, F Nyberg, VK Kuchroo, M Wahren-Herlenius
J Immunol 2006; 176: 6277-6285.

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LIST OF ABBREVIATIONS

ACLE	Acute cutaneous lupus erythematosus
ACR	American College of Rheumatology
ADCC	Antibody-dependent cell mediated cytotoxicity
ANA	Antinuclear antibody
APC	Antigen presenting cell
C	Complement
CCLE	Chronic cutaneous lupus erythematosus
CD	Clusters of differentiation
CHB	Congenital heartblock
CLA	Cutaneous lymphocyte-associated antigen
CLE	Cutaneous lupus erythematosus
DLE	Discoid lupus erythematosus
DLP	Dust-like particles
ELISA	Enzyme linked immunosorbent assay
HLA	Human leukocyte antigen
HMGB1	High mobility group box chromosomal protein 1
hYRNA	Human cytoplasmic ribonucleic acid
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
kD	kilo Dalton
LE	Lupus erythematosus
LBT	Lupus band test
MCTD	Mixed connective tissue disorder
MHC	Major histocompatibility complex
NLE	Neonatal lupus erythematosus
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PLE	Polymorphous light eruption
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
SCLE	Subacute cutaneous lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Sjögren's syndrome
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TNF	Tumor necrosis factor
TRIM	Tripartite motif
Ub	Ubiquitin
UCTD	Undifferentiated connective tissue disorder
UV	Ultraviolet
VCAM	Vascular adhesion molecule

1 BACKGROUND

Lupus erythematosus (LE) is an autoimmune disease characterized by a wide variety of clinical manifestations ranging from cutaneous lesions to multiorgan involvement. The term lupus, meaning wolf in Latin, was first used as a medical description of cutaneous lesions resembling a wolf's bite. In the middle of the 19th century Cazenave introduced the term "lupus erythemateux" to distinguish it from cutaneous tuberculosis (lupus vulgaris), and von Hebra used the term butterfly to describe the malar rash of the disease (reviewed in Rowell 1997; Wallace and Lyon 1999). In 1872 Kaposi recognized the systemic visceral symptoms of the disorder and proposed two types of diseases: the discoid and the disseminated (systemic) forms (Kaposi 1872). This was further supported by Osler, who in the late 1890's described the visceral involvement of lupus in detail (Osler 1895). The serological and immunological manifestations found in lupus patients were described in the 20th century in the form of false positive test results for syphilis, discovery of the LE cell phenomenon (phagocytes which have phagocytized nuclei) (Hargraves et al. 1948) and the detection of anti-nuclear antibodies (ANA) (Friou et al. 1958). Further studies of immunological abnormalities resulted in the detection of more specific autoantibodies.

Lupus can affect virtually any organ of the body including joints, skin, lungs, kidneys and blood. In 1982 the American College of Rheumatology (ACR) set the criteria for classification of systemic LE (SLE), Table 1 (Tan et al. 1982). Mucocutaneous manifestations account for four of the 11 ACR criteria, namely butterfly rash, discoid lupus, photosensitivity and oral ulcers. Cutaneous LE (CLE), can be the first sign in patients with onset of SLE, and approximately 70% of SLE patients present cutaneous manifestations sometime during the course of their disease.

Table 1. ACR classification criteria for SLE, adapted from Tan et al (Tan et al. 1982). Four of 11 criteria are needed for SLE diagnosis.

Criterion	Definition
1. Malar rash	Fixed erythema over the malar eminences
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulcers
5. Arthritis	Nonerosive arthritis involving two or more peripheral joints
6. Serositis	Pleuritis or pericarditis
7. Renal disorder	Persistent proteinuria or cellular casts
8. Neurologic disorder	Seizures or psychosis
9. Hematologic disorder	Hemolytic anemia, leukopenia, lymphopenia or thrombocytopenia
10. Immunologic disorder	Positive LE cell preparation, anti-dsDNA antibody, anti-Sm antibody or false positive test for syphilis
11. Antinuclear antibody	Abnormal titer of ANA

1.1 CUTANEOUS LUPUS ERYTHEMATOSUS

Patients with CLE are often photosensitive and the cutaneous lesions can both be induced and aggravated by the sun (Lehmann et al. 1990). The lesions which are often distributed over sun-exposed areas of the body can remain for weeks, or even months and can heal with scarring. The skin lesions of LE may present as a malar rash, as annular/papulosquamous non-scarring lesions or as a discoid plaque healing with scarring and atrophy. Although the majority of the patients with CLE have a relatively mild disease course, some patients are at risk of developing SLE.

1.1.1 Classification

Classification enables investigation and comparison of possible systemic features in the different variants of CLE. Thus, the frequency of SLE for each subtype of CLE can be evaluated. Classification is also important for educational purposes for patients and in health care. Gilliam and Sontheimer have classified the cutaneous lesions found in LE patients (Gilliam 1977; Gilliam and Sontheimer 1981). This classification is currently the most accepted and frequently used, and subdivides the cutaneous lesions of LE into those with LE-specific histology (LE-specific) and those without LE-specific histology (LE-nonspecific), Table 2.

Table 2. Classification of cutaneous lesions in LE (Gilliam 1977; Gilliam and Sontheimer 1981).

LE-specific	***LE-nonspecific***
Acute cutaneous LE	Cutaneous vascular disease
Localized ACLE	Alopecia (nonscarring)
Generalized ACLE	Sclerodactyly
Subacute cutaneous LE	Rheumatoid nodules
Annular	Calcinosis cutis
Papulosquamous	LE-nonspecific bullous lesions
Chronic cutaneous LE	Urticaria
Classical DLE	Papulonodular mucinosis
Localized DLE	Anetoderma/cutis laxa
Generalized DLE	Acanthosis nigricans
Hypertrophic/verrucous DLE	Erythema multiforme
Lupus panniculitis/profundus	Leg ulcers
Mucosal LE	Lichen planus
Lupus tumidus	
Chilblains lupus	

1.1.2 LE-specific skin lesions

According to the classification by Gilliam and Sontheimer (Gilliam 1977; Gilliam and Sontheimer 1981) the LE-specific skin lesions are classified into: acute CLE (ACLE),

subacute CLE (SCLE) and chronic CLE (CCLE). In most patients, one of these subsets of CLE normally predominates, but overlap occurs between the subsets and a patient can display more than one CLE subset which makes epidemiological studies difficult to perform. LE-specific skin lesions have a histopathological picture characteristic for LE.

1.1.3 Histopathology

The characteristic histopathological finding of LE-specific skin lesions is the lichenoid tissue reaction pattern i.e. damage of the dermal-epidermal junction and dermal mononuclear cell infiltrates (Lever and Schaumburg-Lever 1997). The principal site of injury is the basal layer of the epidermis which is seen as a hydropic degeneration of the epidermal basal cell layer and a thickening of the basal membrane. Apoptotic keratinocytes, also known as civatte bodies, are present in the epidermis. In addition, the epidermis displays atrophy and hyperkeratosis sometimes filling the follicular openings to form the characteristic "plugging". Close to the basal keratinocytes undergoing apoptosis and cytotoxic injury, a mononuclear cell infiltrate is seen in the upper dermis, and in the periappendageal and perivascular areas. This infiltrate is composed of infiltrating T cells and macrophages (David-Bajar and Davis 1997). A few plasma cells may also be seen in the dermis. The infiltrating T lymphocytes consist of more CD4+ or helper T cells than CD8+ or cytotoxic T cells (Tebbe et al. 1995; Kuhn et al. 2002). In the dermis abundant mucin is often seen, the substance is produced by fibroblasts which have been demonstrated to have an impaired proliferation rate in scarring forms of CLE (Nyberg et al. 2000). Distinction of the histopathology in the different subsets of lupus is difficult without prior knowledge of the clinical and serological findings. However, follicular plugging and hyperkeratosis tend to be more prominent in CCLE lesions, and the inflammatory infiltrate in CCLE is more dense and infiltrates deeper into the dermis than in SCLE (Bangert et al. 1984; David-Bajar and Davis 1997).

1.1.4 Immunofluorescence

In the 1960's deposits of immunoglobulins and complement components were found in the dermal-epidermal junction in LE patients using direct immunofluorescence techniques (Burnham et al. 1963; Kalsbeek and Cormane 1964). These deposits were termed the lupus band. The deposits, however, are also present in healthy skin of SLE patients (Cormane 1964) as well as in sun-exposed skin from normal healthy subjects (Fabre et al. 1991). The significance of a positive lupus band test (LBT) is debated and the clinical implications of a positive LBT are difficult to evaluate. Studies have shown that the presence of IgG combined with two additional Ig subtypes in non-lesional skin (non-lesional LBT) is more specific for SLE (Velthuis et al. 1992).

Another finding in immunofluorescence staining are the dust-like particles (DLP). These DLPs are seen as a specific direct immunofluorescence staining pattern scattered primarily in the epidermis, of CLE patients with Ro/SSA autoantibodies (Nieboer et al. 1988; Nyberg et al. 1998). This pattern can be reproduced experimentally by infusing anti-Ro/SSA autoantibodies into human skin-grafted mice (Lee et al. 1989), indicating that DLPs represent the binding of immunoglobulins to the Ro antigen in vivo.

1.1.5 Acute cutaneous lupus erythematosus

ACLE is most often presented as a butterfly-shaped facial eruption (erythema or maculopapules) localized to the malar regions sparing the nasolabial folds. ACLE may also be a generalized skin condition. This subset of LE is almost always found in patients with active SLE and is therefore often seen in rheumatology departments.

1.1.6 Subacute cutaneous lupus erythematosus

SCLE was originally described as a distinct subset of LE by Sontheimer, Thomas and Gilliam in 1979 (Sontheimer et al. 1979) and after a 10-year follow-up, was confirmed to be a rather homogenous subgroup within the spectrum of lupus (Callen and Klein 1988). Prior to 1979 descriptions of "symmetric erythema centrifugum", "disseminated LE" and "Rowell's syndrome" show similarities to the symptoms described by Sontheimer et al (Rowell et al. 1963; Sontheimer et al. 1979). SCLE is clinically characterized by pronounced photosensitivity, i.e. induction of new skin lesions or exacerbations of existing skin lesions upon exposure to ultraviolet (UV) radiation (Kuhn et al. 2001b). The lesions of SCLE (Figure 1) are typical erythematous non-scarring lesions distributed symmetrically over the sun-exposed areas of the body, such as the V area of the upper chest and upper back and extensor surfaces of the upper extremities. The morphological types of lesions may be annular or papulosquamous, and patients may also have a combination of both types. Annular lesions have a central clearing and often a slight scale, and may be mistaken for erythema multiforme or erythema annulare centrifugum. The papulosquamous variant presents scaly plaques and can simulate psoriasis or lichen planus. The lesions of SCLE may last for weeks and as the lesions resolve, postinflammatory hypopigmentation can result along with telangiectasias.

Approximately 50% of SCLE patients fulfil at least four of the ACR diagnostic criteria for SLE. (Sontheimer et al. 1982; Tan et al. 1982; Callen and Klein 1988). SCLE patients fulfilling the ACR criteria often have a relatively benign form of SLE marked by musculoskeletal symptoms. These patients rarely develop systemic manifestations such as renal or central nervous system disease (Chlebus et al. 1998; Parodi et al. 2000).

SCLE is closely associated with the presence of Ro/SSA autoantibodies (Sontheimer et al. 1982). These autoantibodies occur in a frequency of about 70% but figures vary depending on which method is used for detection (Sontheimer et al. 1982; Lee et al. 1994; Chlebus et al. 1998). Besides Ro/SSA autoantibodies, SCLE is also associated with the presence of HLA-DR3 (Sontheimer et al. 1982; Johansson-Stephansson et al. 1989). Ro/SSA-positive SCLE can be induced by certain drugs e.g. anti-hypertensives and anti-fungal agents, and is then referred to as drug-induced SCLE. Drug-induced SCLE skin lesions have been described to resolve following withdrawal of the drug (Reed et al. 1985; Srivastava et al. 2003). Whether the Ro/SSA autoantibodies are induced by the drug or are present before exposure to the drug has not previously been studied prospectively.

1.1.7 Chronic cutaneous lupus erythematosus

CCLE is the most common subtype of CLE. There are different forms of CCLE (Table 2); the classical discoid LE (DLE) (localized and generalized), hypertrophic/verrucous DLE, lupus panniculitis/profundus, mucosal LE, lupus tumidus and chilblains lupus. The classical DLE (Figure 1) is the most common form consisting of fixed, indurated erythematous scaly plaque, mainly located on light exposed areas such as the face and scalp healing with scarring and atrophy. DLE lesions might also be found in sun-protected areas like the hair-bearing scalp. Scarring alopecia has been found in 33% of patients with DLE, further adding to their social handicap (Wilson et al. 1992).

Approximately 5-10% of the patients with classical DLE will develop SLE (Millard and Rowell 1979; Healy et al. 1995) usually more than five years after the onset of the cutaneous lesions (Le Bozec et al. 1994; Healy et al. 1995). Patients with high ANA titers and/or patients with generalized DLE (i.e. lesions above and below the neck) tend to be more frequently associated with SLE (Prystowsky and Gilliam 1975; Millard and Rowell 1979; Tebbe and Orfanos 1997). CCLE is not normally associated with autoantibodies, but depending on the sensitivity of the serological method used, low titers of anti-Ro/SSA may be detected (Lee et al. 1994).

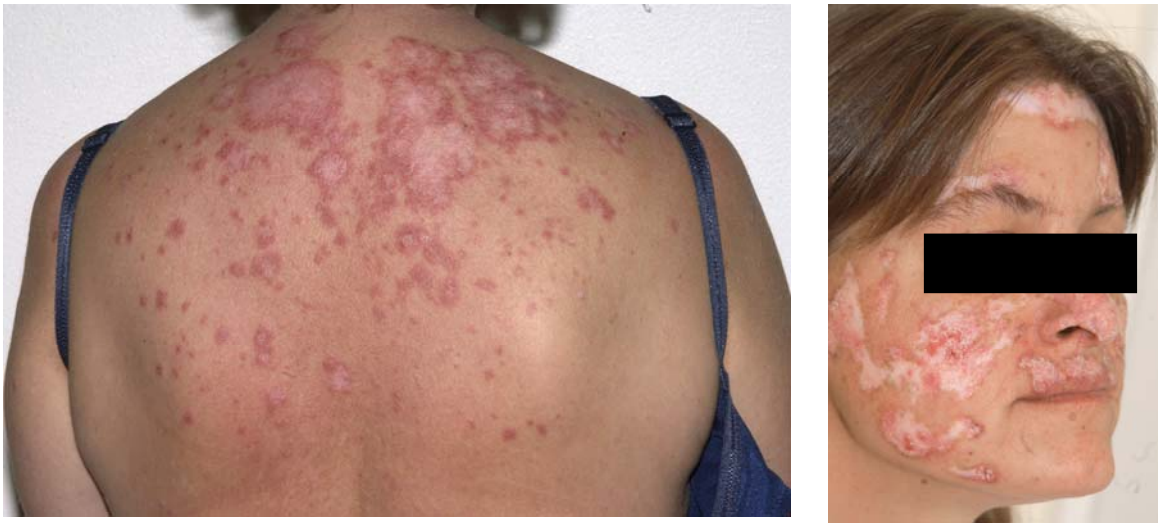

Figure 1. Cutaneous manifestations of SCLE (left) and DLE (right).

1.1.8 LE-nonspecific skin lesions

LE-nonspecific skin lesions are found in LE as well as in other diseases. The LE-nonspecific skin lesions are listed in Table 2 and include lesions such as cutaneous vascular disease (e.g. vasculitis, livedo reticularis, thrombophlebitis, Raynaud's phenomenon), nonscarring alopecia, leg ulcers, urticaria and bullous lesions. The possible prognostic implications associated with such nonspecific skin lesions in patients with CLE, have not been investigated.

1.2 THE IMMUNE SYSTEM AND AUTOIMMUNITY IN CLE

The striking dermal mononuclear cell infiltrates, autoreactive antibodies in serum, lack of pathogenic microorganisms and clinical response to immunosuppressive or modulating drugs implicate cutaneous lupus as an autoimmune disease.

The immune system is a network of many different components with a common goal to defend the body against foreign pathogens. The immune system normally only responds to foreign pathogens but can also start an immune reaction against endogenous antigens leading to autoimmunity. Autoimmunity is the result of a breakdown of tolerance or unresponsiveness to self-antigens. Immunological tolerance can be divided into central and peripheral tolerance and failure of either of these mechanisms may result in the development of autoimmune disease. Autoreactive cells are also found in individuals without autoimmune disease. Why autoreactive cells are silent in some individuals but not in others is not fully understood, but different factors such as genetic susceptibility as well as exogenous factors have been suggested to be involved.

Autoimmune disorders occur in approximately 5% of the population in the Western world and have traditionally been classified into those mediated by T cells and those mediated by B cells. It has recently been suggested that both T cells and autoantibodies cause tissue damage, and thus the separation of autoimmune disease into by B cells or T cells mediated should perhaps not be done (Davidson and Diamond 2001). Today there are over 40 different human diseases, classified as either definite or probable autoimmune disease. Autoimmune diseases can be systemic e.g. SLE, and/or organ-specific e.g. Hashimoto's thyreoiditis. CLE represents an autoimmune disease with features of both systemic and organ specific disease.

The frequent finding of autoantibodies implicates that the autoimmune reaction in CLE is typically directed to the Ro/SSA and La/SSB antigens. These antigens are found intracellularly in all nucleated cells. The Ro/SSA antigen consists of a 52 kD (Ro52) and a 60 kD (Ro60) component, where the Ro60 can bind to small RNAs, hYRNAs. The Ro52 protein does not bind RNA, and while early studies indicated binding of the protein to the Ro/SSA particle (Ben-Chetrit et al. 1988) later investigations have been unable to confirm the presence of Ro52 in the Ro60/hYRNA complex (Kelekar et al. 1994; Boire et al. 1995). Rather, a role for Ro52 as an E3 ligase has been suggested (Espinosa et al. 2006; Sabile et al. 2006; Wada and Kamitani 2006). Ro60 recognizes defective RNAs that are eventually targeted for degradation (reviewed in Chen and

Wolin 2004). The SSB antigen contains the La 48 kD protein. La binds RNA polymerase III transcripts, and is involved in maturation of the transcripts (Gottlieb and Steitz 1989). Ro60 and La resides predominantly in the nucleus but both proteins may shuttle to the cytoplasm (Simons et al. 1994). The major protein moiety of Ro52 is found in the cytoplasm (Simons et al. 1994; Pourmand et al. 1998).

1.3 A MODEL FOR ETIOPATHOGENESIS

Although the exact etiology of lupus is unknown, genetic, hormonal and environmental factors contribute to the development of the disease. In a genetically predisposed patient, an environmental factor could trigger the immune system resulting in the development of the disease. Currently known possible risk factors that may influence or trigger an immune reaction in lupus patients are illustrated in Figure 2.

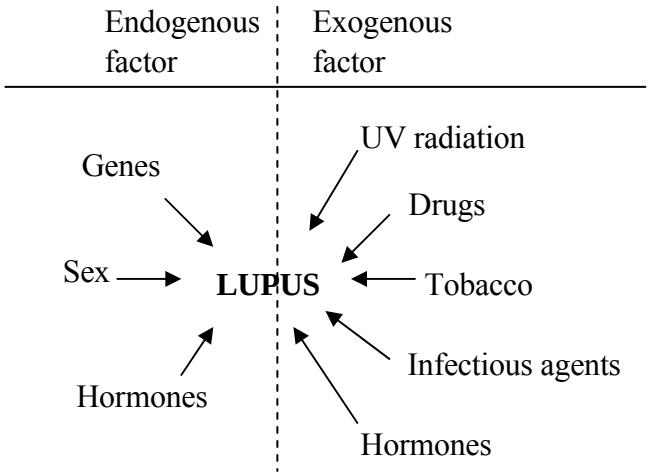

Figure 2. Risk factors associated with lupus

A model including four phases has been used to describe the pathogenesis of CLE (Costner et al. 2004).

1.3.1 Susceptibility phase

The first phase is called the susceptibility phase and involves the genetic susceptibility to CLE. Familial clustering has been found in LE which supports the idea of a genetic role as a risk factor for lupus. In one study, significantly increased prevalence of DLE was reported in first-degree relatives of DLE patients compared with controls (Lawrence et al. 1987). The heritability analysis in the same study suggested a polygenic inheritance with a heritability of 44%. Reports have also described the occurrence of DLE in monozygotic twins (Steagall et al. 1962; Kohler et al. 1974;

Wojnarowska 1983). There are no concordance figures for CLE but studies of SLE have shown a 24-57% concordance in monozygotic twins (Block et al. 1975; Deapen et al. 1992) and a 2-5% concordance in dizygotic twins and siblings (Deapen et al. 1992).

1.3.1.1 HLA genes

An association between LE and certain major histocompatibility complex (MHC) or human leukocyte antigen (HLA) genes has been observed. The HLA family of genes is located on the short arm of chromosome 6. They are organized into three regions which encode HLA class I (HLA-A, –B, -C), HLA class II (HLA-DP, -DQ, -DR) and HLA class III (complement components, TNFα, and heat shock proteins) molecules. The multiple genes and large polymorphism in this gene family allows for varied expression and many autoimmune diseases are associated with certain HLA subtypes. Genes in the HLA class II group are linked to lupus especially the DR2 and DR3 variants and increased frequency of HLA-DR2 and HLA-DR3 have been found in SLE patients (Ahearn et al. 1982). An increased frequency of HLA-DR3 expression has also been found in patients with Sjögren's syndrome (SS) (Wilson et al. 1984). When Ro/SSA-positive patients are removed from the groups of SLE and SS patients, no association between HLA-DR2 and HLA-DR3 is found, suggesting that HLA-DR2 and HLA-DR3 are associated with Ro/SSA production rather than the phenotypic expression of the disease (Provost and Watson 1993; Gottenberg et al. 2003). Taken together, the capacity of an individual to present Ro/SSA antigen to the lymphocytes is determined by the specificity of HLA. In one study, the presence of both HLA-DQ1 and DQ2 led to the highest titers of anti-Ro/SSA suggesting that MHC or HLA also determine the level of the response (Harley et al. 1986). As proposed by Provost et al, anti-Ro/SSA HLA-DR3 positive women with SS, SCLE or SS/LE overlap, or those who are asymptomatic are immunogenetically closely related and children of mothers from all of these groups are at risk of being born with neonatal LE (NLE) (Provost and Watson 1993).

The HLA-A1, B8, DR3, DQ2, DRw52 and C4null ancestral haplotype has been linked to SCLE, especially in Ro/SSA-positive patients (Sontheimer et al. 1981; Sontheimer et al. 1982; Sontheimer 1987; Johansson-Stephansson et al. 1989). CCLE has been associated with A1, B8, DR3 haplotypes despite negative autoantibody serology and the authors proposed a broader role for HLA in disease pathogenesis than has previously been proposed (Millard et al. 2001b).

1.3.1.2 Complement genes

The complement system is part of the immune system. In short, the complement components are involved in the clearance and thereby also the detoxification of immune complexes and apoptotic cells after an autoimmune attack has occurred. Apoptotic bodies are rich in autoantigens and without the help of complement in clearance, the concentration of autoantigens will rise and subsequently be exposed to the antigen presenting cells (APC) resulting in abnormal cellular or humoral response. The complement components are encoded by the genes of the class III region which map between HLA class I and II. Deficiencies in the proteins coded by C4 and C2

genes, leading to defects in the clearance of immune complexes and apoptotic cells, have been linked to SCLE and DLE (Meyer et al. 1985; Braathen et al. 1986; Callen et al. 1987).
C1q genes are located on chromosome 1. Homozygous C1q genetic deficiency is a strong genetic risk factor for the development of SLE. C1q is an important physiological factor in the removal of apoptotic cells and has been found to bind to surface blebs of apoptotic human keratinocytes (Korb and Ahearn 1997) thereby promoting the clearance of apoptotic cells. A genetic deficiency of C1q with corresponding decreased serum levels of C1q has been associated with SCLE (Racila et al. 2003).

1.3.1.3 Non-HLA genes

Other genes that have been associated or appear to confer susceptibility to CLE or Ro/SSA antibody production include the genes encoding IL-1 (2q13), IL-10 (1q31), TCR (Cβ1, Cβ2) (7q35), Fc gamma receptor (FcγRII) (1q23), intracellular adhesion molecule (ICAM) (19p13.3-p13.2), E-selectin (1q23-25), and Fas (10q24.1) (reviewed in Millard and McGregor 2001). Abnormal expression of cytokines and adhesion molecules are found in CLE patients and previous studies have demonstrated an increased expression of ICAM-1 and E-selectin in the skin and serum implying abnormal regulation of the genes encoding these proteins (Stephansson and Ros 1993; Nyberg et al. 1997a; Nyberg et al. 1999). Other non-HLA genes known to be associated with SLE, and that may be associated with CLE are protein tyrosine phosphatase 22 (PTPN22) (1p13), Cytotoxic T-lymphocyte antigen 4 (CTLA4) (2q33), interferon regulatory factors 5 and 3 (IRF5 and IRF3) (7q32 and 19q13.3) and Toll-like receptor 5 (TLR5) (1q41) (reviewed in Wong and Tsao 2006). It is likely that these genes contribute to disease susceptibility in varying degrees in different patients or subsets of disease. It is also striking that many of these genes have been identified as susceptibility factors in several other autoimmune conditions.

1.3.2 Induction phase

Induction or initiation of autoimmunity with resulting loss of self-tolerance is the proposed second phase. This phase involves exposure to different environmental factors. UV induced keratinocyte apoptosis is thought to be one of the main initiators of the autoimmune reaction cascade. UVB induced apoptosis of keratinocytes also occurs as a normal event in healthy individuals (reviewed in Zhuang et al. 2000). An increased number of apoptotic cells is found in the epidermis in CLE lesions (Chung et al. 1998; Baima and Sticherling 2001). The increased number of apoptotic keratinocytes might either be due to increased formation or to a decreased clearance of the cells, eventually leading to breakdown of self tolerance. A correlation has been found between reduced uptake of apoptotic cells by macrophages and decreased complement levels in SLE (Bijl et al. 2006). In a recent study increased numbers of apoptotic cells was seen in primary and UV-induced skin lesions of CLE patients compared with normal healthy controls (Kuhn et al. 2006), supporting the hypothesis of delayed or impaired clearance of apoptotic cells. Baima and Sticherling studied apoptosis-related markers and found downregulation of Bcl-2 expression in the basal layer of the epidermis of LE resulting

in susceptibility of the keratinocytes to Fas-dependent apoptosis (Baima and Sticherling 2001). They further demonstrated that the apoptotic rate increases with the acute course of the disease, thus a higher apoptotic rate was found in SCLE skin lesions than in CCLE skin lesions.

In a study by Casciola-Rosen et al, keratinocytes became apoptotic within a few hours after UVB irradiation (Casciola-Rosen et al. 1994). In the same study it was also found that autoantigens are clustered to surface blebs or apoptotic bodies on the surface of apoptotic keratinocytes. Expression of Ro/SSA antigens in these blebs can be induced by UVB (LeFeber et al. 1984; Furukawa et al. 1990; Golan et al. 1992; Jones 1992b; Zhang et al. 2000) and by UVB induced epidermal cytokines (Dorner et al. 1995). Other factors mediating surface expression of Ro/SSA antigen on keratinocytes include estrogen (Furukawa et al. 1988; Jones 1992a; Zhang et al. 2000), viral infection (Zhu 1995; Zhu 1996) and heat shock (Zhang et al. 2000).

1.3.3 Expansion phase

Phase III involves the expansion of T cells and autoantibody formation. Exposure to UV radiation induces the release of proinflammatory cytokines which in turn leads to expression of adhesion molecules and selectins on keratinocytes and lymphocytes. This results in migration of lymphocytes through the endothelial barrier into the skin (Bennion and Norris 1997). The infiltrating T cells have been suggested to be CD4+ T cells, and less CD8+ T cells are seen (Tebbe et al. 1995; Kuhn et al. 2002). The apoptotic derived antigenic material is phagocytized by dendritic cells which present the antigens to both MHC class II and MHC class I restricted T cells. CD4+ T cells interact with B cells promoting autoantibody production while the CD8+ T cells mediate cellular cytotoxic mechanisms.

1.3.4 Injury phase

Phase IV is the injury phase. In SCLE it has been suggested that circulating autoantibodies bind to keratinocytes expressing surface autoantigens. This leads to destruction of the keratinocytes through antibody-dependent cell mediated cytoxicity (ADCC) by the infiltrating lymphocytes (Norris 1993). Furukawa et al observed that cultured keratinocytes from patients with SCLE and SLE showed greater cytotoxicity and greater susceptibility to ADCC when irradiated with UV (Furukawa et al. 1999). It is not yet known if the autoantibodies play a direct role in the pathogenesis of SCLE since not all patients with Ro/SSA autoantibodies develop SCLE and about 30% of SCLE do not display Ro/SSA autoantibodies in sera (Chlebus et al. 1998). Nevertheless, an indication that these autoantibodies are pathogenic comes from the observation that in NLE, where the child acquires autoantibodies from the mother, the child presents skin lesions equivalent to SCLE. These lesions disappear at the time that maternal autoantibodies are cleared. In CCLE autoantibody induction of keratinocyte damage is less likely to occur, and instead a specific subset of gamma delta receptor positive T cells was thought to be the effector of keratinocyte lysis in CCLE (Volc-Platzer et al. 1993) but these findings have not been reproduced (Fivenson et al. 1991). Recently, a subset of circulating memory T cells have been found that selectively

localize in cutaneous sites mediated in part by the interaction of cutaneous lymphocyte-associated antigen (CLA) with its vascular ligand E-selectin (Berg et al. 1991). A large number of skin-homing cytotoxic lymphocytes co-expressing CLA have been found in DLE and are thought to play an important role in the pathogenesis of DLE (Wenzel et al. 2005a). Thus, the pathogenic mechanisms of SCLE and CCLE may differ partially, since cytotoxicity is proposed to be mediated by autoantibodies in SCLE and T cells in CCLE.

Taken together, interplay of various factors can thus result in immune reactions directed against self, resulting in tissue damage with the final development of an autoimmune disease. By studying clinical and serologic findings but also etiopathogenic factors contributing or taking part in the pathogenesis of lupus patients we can increase our understanding of how an autoimmune disease is initiated and how tissue injury is mediated. Although lupus is a heterogenous disease that may affect any organ of the body, study of the pathogenesis in skin biopsy specimens is an attractive model because it offers direct access to the affected tissue.

2 THE PRESENT INVESTIGATION

2.1 AIMS OF THE THESIS

Cutaneous lupus erythematosus is a common form of lupus, and offers an attractive opportunity to study human autoimmune disease as the lesions are visible and can be experimentally induced by known external factors. It is therefore possible to follow the disease longitudinally from eruption to healing. The lesions are also readily accessible for biopsy to study the cellular and molecular events of the disease. Cutaneous lupus primarily affects women, is often not optimally treated and may be socially handicapping. By studying clinical and immunological features in CLE and in Ro/SSA-positive patients we hope to contribute to a better understanding of lupus and as well as many related autoimmune diseases. We aim to offer new insight into the molecular pathways involved in the pathogenesis of the disease, hopefully leading to new treatment options for the patients. This thesis includes characterization of the clinical, serological and molecular findings in CLE and in Ro/SSA-positive patients, with the specific aims:

- To define the incidence and prevalence of Ro/SSA-positive SCLE

- To describe the fine specificity of the Ro/SSA autoantibodies in correlation to the clinical picture, disease activity and environmental factors

- To evaluate disease progression in a short-term perspective of Ro/SSA-positive patients

- To study the autoimmune reaction in the target tissue, and to identify key cytokines in the pathogenesis of CLE

- To study the expression and function of Ro52

2.2 EPIDEMIOLOGY OF LUPUS ERYTHEMATOSUS

It is important to evaluate epidemiological data such as incidence and prevalence, in order to allocate sufficient resources for the care of the patients and for planning and design of clinical trials. Figures on frequency and distribution are also important to increase awareness about a patient group with rare diseases, to meet their needs with structured evaluable health care. Also, clarification of causative factors, prognosis and the development of treatment guidelines for the patients rely on an identified patient group where basic epidemiological data are known.

2.2.1 Epidemiology of SLE

Figures on the incidence and prevalence of SLE vary depending on the population studied. In Sweden the incidence of SLE has been estimated to be 4.8 per 100,000 inhabitants and the prevalence to be 39-68 per 100,000 inhabitants (Nived et al. 1985; Stahl-Hallengren et al. 2000). Due to the improved detection of mild disease the incidence of SLE has increased during the past years (Uramoto et al. 1999).

2.2.2 Epidemiology of Ro/SSA-positive SCLE

Population based studies of the epidemiology of CLE are lacking but CLE is believed to be two to three times more frequent than SLE (Tebbe and Orfanos 1997). SCLE was found in 9-21% and CCLE in 42-72% of selected patients with LE seen in dermatology departments (Sontheimer et al. 1979; Kind and Goerz 1987; Tebbe and Orfanos 1987; Weinstein et al. 1987). Since there are no diagnosis-based registers from dermatology outpatient departments in Sweden, and since histopathologists usually do not discriminate between the subtypes of CLE, any patients diagnosed with SCLE cannot be identified by studying diagnosis-based health care registers in Sweden.

We conducted a retrospective study in order to define the incidence and prevalence of SCLE in Stockholm **(Paper I)**. In light of the knowledge that the majority of patients with SCLE have Ro/SSA autoantibodies, we identified all patients in Stockholm county (1.8 million inhabitants) who tested Ro/SSA-positive between the years 1996-2002 (n=1323). A questionnaire was sent to those patients who were still living in Stockholm in 2003 (n=1048), and 741 patients answered this questionnaire. Since we know that the majority of all SCLE patients are photosensitive, we chose to perform an in-depth study of questionnaire responders with self-reported photosensitivity and active skin symptoms. Of these, 125 patients were called to the clinic. These 125 patients constitute the basis for several studies in this thesis. Of the 125 patients, 20 patients fulfilled Sontheimer et al's original description of SCLE (Sontheimer et al. 1979), with sixteen of the cases being incident during the study period. The different study selection steps are illustrated in Figure 3.

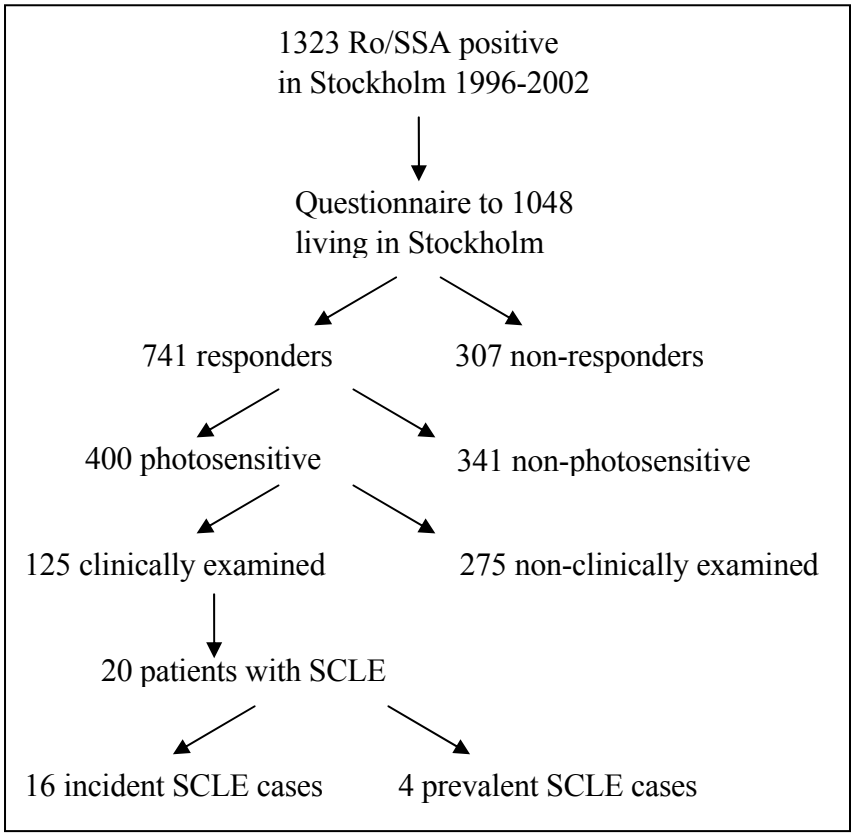

Figure 3. A schematic representation of the different study selection steps.

Estimates of the occurrence of SCLE were based on the following assumptions: an equivalent frequency of SCLE among the not clinically investigated 275 out of 400 patients with self-reported photosensitivity (both with and without active cutaneous symptoms), no SCLE among the 341 out of 741 questionnaire respondents who did not report photosensitivity, and equivalent frequency of photosensitivity and SCLE among the non-responders and among the Ro/SSA-positive patients who had moved from Stockholm or died during the study period. In addition, for those who died during the study period, the person-time value was estimated as an average, representing one-half the study time period (3.5 years).

Estimates of incidence and prevalence revealed an incidence rate for Ro/SSA-positive SCLE of 0.7/100,000 during the years under study. A rough estimate of the prevalence of Ro/SSA-positive SCLE was 6.2-14/100,000 depending on disease duration. Overall incidence and prevalence of all SCLE (with and without the presence of Ro/SSA autoantibodies) was estimated to be 1 per 100,000 persons per year and 8.9-20 per 100,000 persons respectively based on an estimate of 70% Ro/SSA-positive patients. The figures include SCLE patients both with and without SLE. In Table 3 epidemiological data of other autoimmune diseases are shown for comparison.

Table 3. Epidemiological figures of autoimmune disorders.

Autoimmune disease	Incidence/100,000
Adult rheumatoid arthritis (Jacobson et al. 1997)	23.7
Systemic lupus erythematosus (Nived et al. 1985)	4.8
Primary Sjögren's syndrome (Plesivcnik Novljan et al. 2004)	3.9
Subacute cutaneous lupus erythematosus	1

SCLE has been reported in 9-21% of patients with LE who were seen in dermatology departments (Sontheimer et al. 1979; Weinstein et al. 1987) i.e. CCLE is five to ten times more common than SCLE in most clinical settings. Extrapolation of this relationship to the population at large, would give a prevalence of 45-89/100,000 persons, and the prevalence of SLE in our country is 39-68/100,000 persons (Nived et al. 1985; Stahl-Hallengren et al. 2000).

To cross examine our results a histopathology-based register study has been initiated and all histopathology samples from all patients diagnosed with CLE between 1996-2002, including our cohort of Ro/SSA-positive patients, are available for investigation and re-examination. The preliminary results that have been obtained from this cross-over register study largely confirm our present results. The preliminary results applied to our cohort show that SCLE was found less frequently among non-responders (4.6%) compared to responders (6.6%) in contrast to one of our assumptions of equal frequency. The reason for this finding might be that responders are sicker than non-responders and therefore are more likely to answer the questionnaire. Our assumption that there should be no SCLE cases among patients with no self-reported photosensitivity was not entirely confirmed by the register, which revealed a few cases of SCLE in this group as well. There was also a difference in the frequency of SCLE among patients with self-reported photosensitivity who underwent clinical examination, and those who did not, and this difference is probably due to the fact that all of the patients who were examined clinically also reported active skin symptoms. Perhaps those with active skin symptoms also are more likely to answer the questionnaire than those not having active skin symptoms. In conclusion, the preliminary results of the cross-over study indicate slight differences but overall they support our findings. The preliminary results reveal 63 cases with a histopathological diagnosis of SCLE over a seven year time period, a figure which supports our results. Since the SCLE and CCLE can not be distinguished in all cases, the cross-over study has limitations but almost all the histopathology samples were examined by experienced dermatopathologists who usually attempt to distinguish between the subsets. Taken together, the preliminary results obtained from the cross-over study largely show that almost all of SCLE cases are indeed found in the group of patients that were selected from the questionnaire.

In order to further cross check our data we estimated the incidence and prevalence of SLE in our cohort. If the same assumptions as discussed above are used, and if we assume that 30% of the SLE patients are Ro/SSA-positive, the incidence was estimated to 3/100,000 persons per year and the prevalence was estimated to 43/100,000. These findings clearly support our method of calculation since it comes surprisingly close to

the established SLE incidence and prevalence in Sweden as well as in other countries. If the estimated prevalence of SCLE and CCLE subsets and SLE in our study are compared, CLE is 1.2-2.3 times more common than SLE, thus a little less than the ratio of CLE to SLE, which has earlier been suggested to be 2-3:1 (Tebbe and Orfanos 1997).

The advantage of the serology-based epidemiological approach in Paper I is that we identified all Ro/SSA-positive patients including Ro/SSA-positive SCLE cases forming a cohort. From this cohort we were able to identify the Ro/SSA-positive SCLE patient group, as well as other autoimmune diseases associated with autoantibodies. Collection of clinical data and biobanking purified products from blood and skin biopsy samples could be performed for further experimental research. Because a laboratory test can not be used solely for case-definition all our cases were defined after clinical examination. Since our goal was to find as many SCLE patients as possible we used a questionnare. Our hypothesis was that 70% of SCLE patients in a defined population will be found among Ro/SSA positively tested patients with a history of photosensitivity and skin lesions. Using the questionnaire as a method to find patients with CLE and self-reported photosensitivity has previously been validated in a large Scandinavian study (Nyberg et al. 1997b). In that study only 20% of CLE patients self-reported no photosensitivity.

In our papers we have consistently used the classification system of Gilliam and Sontheimer. Previous studies have shown that SCLE (even in cases fulfilling four or more ACR criteria for SLE diagnosis) is a different subset with milder systemic disease, serological differences and higher frequency of photosensitivity compared to SLE patients (Chlebus et al. 1998). A problem when following the patient cohort for several years, is that the patient might move from one diagnostic group to another thus making the determination of prevalence less reliable. To solve this problem our patients with SCLE were always assigned to the SCLE group regardless of whether they fulfilled four or more ACR criteria or not.

2.3 CLINICAL CORRELATES OF Ro/SSA AUTOANTIBODIES

2.3.1 Ro/SSA autoantibodies in CLE

We used Ro/SSA serology as the primary basis for our patient selection. These autoantibodies have been found in increased frequency in several autoimmune diseases and were first associated with SLE and SS (Clark et al. 1969; Alspaugh and Tan 1975). Approximately 70% of SS patients and 30-40% of SLE patients have been found to carry these autoantibodies. In mothers of babies born with congenital heart block (CHB) the autoantibodies are found in almost 100% of the cases. In SCLE the autoantibodies are found in approximately 70% of the patients, but the rate varies depending on which method is used for detection (Sontheimer et al. 1982; Lee et al. 1994; Chlebus et al. 1998). The immune response to Ro/SSA and La/SSB is heterogenous. Studies of the autoantibody binding of particular epitopes of Ro/SSA and La/SSB have shown associations with different autoimmune diseases. Anti-Ro52 is

more common in patients with primary SS (pSS) while both anti-Ro52 and anti-Ro60 are more frequent in SLE patients (Lopez-Longo et al. 1994). The fine specificity of Ro/SSA and La/SSB might provide insights into the pathogenesis and serve as a clinical tool in the differentiation between different autoimmune diseases and can thus be used for diagnostic purposes. An association between autoantibody levels and clinical disease activity has also been observed in patients with SS and SLE (Wahren et al. 1998).

The different diagnoses of the 125 clinically examined Ro/SSA-positive patients (**Paper I**) are described in Table 4. We also found Ro/SSA-positive subjects with no diagnosis (n=9). Previous studies have found Ro/SSA antibodies in 0.2-0.44% of asymptomatic healthy blood donors (Fritzler et al. 1985; de Vlam et al. 1993).

Table 4. Clinical data from 125 Ro/SSA-positive patients (**Paper I**)

Diagnosis	Patients (n)	SSA- (n)	PLE (%)
SCLE	20	4	45
CCLE	6	2	83
SLE	33	4	67
Primary SS	22	0	73
Other			
UCTD	25	4	60
RA or MCTD with secondary SS	6	0	100
RA	2	0	100
Polymyositis	1	0	100
Scleroderma	1		100
No diagnosis	9	8	100

We investigated the fine specificity of the Ro/SSA autoantibodies in 96 patients (**Paper II**) using an enzyme linked immunosorbent assay (ELISA). The highest levels of anti-Ro52 were found in pSS patients, while SLE patients had high levels of both anti-Ro52 and anti-Ro60, which is consistent with other studies (Lopez-Longo et al. 1994). SCLE patients displayed higher levels of both anti-Ro52 and anti-Ro60 compared to CCLE patients.

In a recent study using ELISA, a limited variation in the levels of anti-Ro52, anti-Ro60 and anti-La was found in SLE patients (Hassan et al. 2002). It was concluded that the autoantibody profile is fixed at an early stage in the disease and rarely changes. In contrast to this finding, we observed that 22 of the 120 retested patients were found to be seronegative on the second occasion (**Paper I**). Eight patients of nine without diagnosis (one patient was not retested) were Ro/SSA negative. Furthermore, we found that seroconversion was mainly seen in patients with cutaneous lesions and not in patients with systemic disease, Table 4. We propose that this may be due to the low

levels of Ro/SSA autoantibodies in these patients in the original tests and the use of a possibly less sensitive test at follow-up.

Antibodies to Ro/SSA can be detected by various serological methods, of which immunodiffusion and ELISA are the most commonly used. Routine IF-ANA screening using HEP-2 cells as substrate is a good initial screening method, but antibodies to Ro/SSA are not always detected by this method (Blomberg et al. 2000; Dahle et al. 2004). Immunodiffusion, which is traditionally used, is specific but has low sensitivity and has recently been concluded to be a poor technique for the detection of Ro/SSA autoantibodies (Charles et al. 1992). Other methods proposed for Ro/SSA antibody detection are Western blot and ELISA of which ELISA is the most sensitive (Chretien et al. 1994). Western blot may give false negative results, since only denaturated antigens can be used and this method might therefore not detect all antibodies. The ELISA assay can test natively folded antigens and is also capable of quantifying the actual levels of the autoantibodies. This ability to detect low titers of anti-Ro/SSA may, on the other hand, produce false positive test results and decrease the usefulness of the test and therefore, the result from ELISA assays must always be correlated to the clinical signs and symptoms.

Photosensitivity has also been associated with Ro/SSA autoantibodies and is detected by patient history in 41% of anti-Ro/SSA-positive patients (Simmons-O'Brien et al. 1995) and in approximately 70% of LE patients by detailed interview and/or photoprovocation (Hasan et al. 1997). In our study 54% of the Ro/SSA-positive patients, who filled out our questionnaire (n=741) reported photosensitivity (**Paper I**). Among the clinically investigated photosensitive patients (n=125), 86 patients also reported symptoms consistent with polymorphous light eruption (PLE) (Table 4). Increased occurrence of PLE has been described in patients with CLE (Nyberg et al. 1997b; Millard et al. 2001b). We also found that a majority of SS patients also reported PLE and this warrants the investigation of the incidence of PLE in an unselected group of SS patients. We found no correlation between PLE and fine specificities of anti-Ro/SSA and anti-La/SSB antibodies (**Paper II**). Photosensitivity including PLE thus seems to be associated with Ro/SSA autoantibodies also in patients with no signs of lupus at the time of investigation. On the other hand, the most photosensitive form of cutaneous lupus, lupus tumidus (Kuhn et al. 2001a), is not associated with Ro/SSA autoantibodies implying that the autoantibodies are probably not the only factor causing photosensitivity and PLE. Taken together, the evidence suggests that a detailed history about photosensitivity should be included in the medical evaluation of Ro/SSA-positive patients and councelling about photoprotection should be included in relevant cases. Furthermore, Ro/SSA antibodies in patients with PLE should alert the clinician to investigate for LE and/or SS.

2.4 FACTORS CONTRIBUTING TO THE PATHOGENESIS IN LUPUS

The autoimmune reaction in lupus skin is histologically characterized by lichenoid tissue reaction and cellular infiltrates in which infiltrating T cells and macrophages are found close to the apoptotic and cytotoxically injured epidermal basal keratinocytes. The T cell infiltrates consist of increased numbers of CD4+ T cells and fewer CD8+

positive T cells (Tebbe et al. 1995; Kuhn et al. 2002). This T cell imbalance contributes to abnormal cytokine production and impaired regulation of B cells resulting in autoantibody production (Kammer et al. 2002). Increased amounts of apoptotic cells, also called sunburn cells, as well as cytotoxicity of basal keratinocytes are found in all forms of CLE. The increased numbers of apoptotic keratinocytes seen in lupus serve as a source of antigens. Cells recognizing these antigens express abnormal HLA molecules leading to recognition of autoantigens and immunoreaction. Interaction between genes promoting the autoimmune response and different environmental factors, trigger this autoimmune reaction.

2.4.1 TNF genes

TNFα has been suggested to contribute to the pathogenesis of CLE based on the finding that TNFα is released upon UV exposure (Kock et al. 1990), TNFα is involved in UVB induced apoptosis of keratinocytes (Schwarz et al. 1995; Zhuang et al. 1999; Tsuru et al. 2001), and stimulates surface expression of Ro/SSA antigen on the keratinocytes (Dorner et al. 1995).

TNFα and TNFβ are encoded by HLA class III genes. A TNFα promoter polymorphism (guanine to adenine transition at –308A), also known as TNF2, is associated with high TNFα production (reviewed in Abraham and Kroeger 1999). Werth et al found that fibroblasts transfected with TNFα promoter constructs, following exposure to UVB and in the presence of IL-1α, had an increased transcription activity compared to –308G promoter constructs (Werth et al. 2000). Association between SCLE and the rare TNF –308A allele has also been demonstrated (Werth et al. 2000; Millard et al. 2001a).
Werth et al further found a strong association between TNF2 and HLA-DR3 in SCLE patients and proposed that the enhanced Ro/SSA antigen presentation, due to TNFα induced apoptosis together with the presence of HLA-DR3, stimulates an autoantibody response which triggers SCLE (Werth et al. 2000). There was no difference in the prevalence of the UV irradiation sensitive TNF2 in DLE patients compared with controls (Werth et al. 2000).

We extracted DNA from the peripheral blood mononuclear cells (PBMC) of ten patients with CLE (SCLE n=6, DLE n=4) and performed an analysis of TNFα single nucleotide polymorphisms (SNP) by polymerase chain reaction (PCR) (**Paper IV**). Our results revealed that five of the six patients with SCLE carried the rare TNF –308A allele. We next analyzed TNFα expression in CLE and found no increase in expression of TNFα in the skin in patients carrying the A allele compared to patients with the wild-type TNF1 haplotype. We postulated that there might be a difference between in vivo and in vitro conditions or differences in lapse times between the triggering event and the analysis since the lesions in our study were manifest, spontaneously occuring lesions which was not the case in the study by Werth et al who studied transfected fibroblasts in vitro (Werth et al. 2000).

2.4.2 Environmental factors

Triggering of lupus requires an interplay between genetic and environmental factors as has previously been mentioned. Factors like UV radiation, drugs, tobacco, hormones, and infectious agents have been associated with the onset or exacerbation of CLE lesions.

2.4.2.1 Ultraviolet radiation

Photosensitivity is associated with all subtypes of CLE and it is well known that UV radiation can induce skin lesions and exacerbations of disease in LE patients (Lehmann and Kuhn 2005). Patients with SCLE and SLE are more frequently photosensitive than patients with CCLE as reported in a phototesting study in which UV induced characteristic skin lesions in 63% of patients with SCLE, 60% of patients with SLE and in 45% of patients with DLE (Kuhn et al. 2001b). The photoinduced lesions in the same study were induced in 53% of patients by UVA (320-400 nm) and UVB (290-320 nm), 34% by UVA alone and in 42% by UVB alone. Taken together, LE patients seem to be most sensitive to UVB. Furthermore, Kuhn et al showed in the same study that the pathological reactions in patients with LE appeared within one week (range 1 day to 3 weeks) after irradiation and lasted for approximately 1 week to 2 months. The frequent time delay between the photoprovocation and the development of CLE can explain why patients in the clinical situation may not necessarily associate their disease with exposure to sunlight. This is in contrast to patients suffering from PLE in whom the transient lesions always occur within 24 hours after sun exposure.

2.4.2.2 Drugs

Different drugs can induce or exacerbate lupus. In the classical drug-induced SLE, anti-histone antibodies are found but rarely skin manifestations or Ro/SSA autoantibodies. Drug-induced SCLE is on the other hand characterized by Ro/SSA autoantibodies and photosensitive symmetric annular or papulosquamous lesions that are indistinguishable from idiopathic SCLE. Drug-induced SCLE was first described in 1985 (Reed et al. 1985) and since then a wide range of drugs have been reported to trigger SCLE. The most common drugs known to induce SCLE are antihypertensive drugs such as diuretics (Reed et al. 1985), calcium channel blockers (Crowson and Magro 1997), angiotensin converting enzyme inhibitors (Patri et al. 1985), and anti-fungal agents (Callen et al. 2001). Table 5 illustrates different drugs that have been reported to be associated with SCLE.

Table 5. Drugs reported with SCLE (Srivastava et al. 2003; Sontheimer 2005).

Thiazides	Terbinafine
Spironolactone	Cinnarizine
Diltiazem	Hydroxychloroquine
Nifedipine	Etanercept
Verapamil	Infliximab
Captopril	Naproxen
Pravastatin	Ranitidine
Simvastatin	Omeprazole
Griseofulvin	Penicillamine

In a recent paper Srivastava et al described the occurrence of drug-induced Ro/SSA-positive SCLE in 15 out of 70 patients (Srivastava et al. 2003). In the same study improvement or resolution both clinically and serologically after discontinuation of the drug treatment was seen (Srivastava et al. 2003), and the authors concluded that it is the drug that induces Ro/SSA autoantibodies. This is in contrast with the finding of Reed et al where the Ro/SSA autoantibodies remained in thiazide induced SCLE (Reed et al. 1985). Reed et al speculated that the thiazides could be involved in potentiation of epidermal keratinocyte cytotoxicity either as promoters of Ro/SSA antigen expression, as enhancers of epidermal cytotoxicity through direct phototoxicity or as promoters of Ro/SSA antibody production (Reed et al. 1985).

In our study 33% of the 125 clinically examined Ro/SSA-positive patients reported cutaneous adverse drug reactions most commonly against penicillin (**Paper I**). Among these patients one clinically suspected case of drug-induced SCLE was found. This patient developed SCLE after treatment with felodipine in combination with extensive UV exposure. The lesions did not recur after withdrawal of the drug and the patient still remained Ro/SSA-positive four years after the drug was discontinued. We postulate that the retrospective nature of our study and the use of a questionnaire to recruit the patients led to a preferential identification of the idiopathic type of SCLE. Patients with drug-induced SCLE may have been found among the patients with no self-reported photosensitivity but this group was not clinically investigated and cross examination of histopathological registers confirms that there are very few SCLE cases among these patients. With the prospective approach in **Paper III** 51% of Ro/SSA-positive patients reported new medication during an observation time of two years. Two patients in this study developed drug-induced SCLE after treatment with methotrexate and omeprazole, respectively. Both patients had Ro/SSA autoantibodies for some years prior to the occurrence of the SCLE, an observation that was only possible to determine in a prospective study such as this. In the study performed by Srivastava et al, it is not described whether this was also the case among drug-induced Ro/SSA-positive SCLE patients or if autoantibodies appeared together with the drug-induced lesions.
It is thus important to make a thorough evaluation of the types of medication in Ro/SSA-positive patients, and the clinicians must be made aware of the risk of development of drug-induced SCLE in these patients especially with photosensitizers. Patients should also be informed and made aware of this risk.

2.4.2.3 Tobacco

Tobacco contains more than 4500 substances and has been found to have adverse effects in many diseases including atherosclerosis, Crohn's disease and rheumatoid arthritis (RA), but might also have beneficial effects in some diseases, for example ulcerative colitis and sarcoidosis. In the skin, keratinocytes express nicotinic cholinergic receptors and stimulation of these by nicotine enhances keratinocyte adhesion, differentiation and apoptosis and inhibits keratinocyte migration (Misery 2004). Tobacco consumption may thus contribute to the pathogenesis of different dermatological diseases (reviewed in Freiman et al. 2004).

The effects of cigarette smoke on the immune system are reviewed by Sopori (Sopori 2002). In short, smoking is known to result in leukocytosis and has also been shown to reduce the serum levels of immunoglobulins (Ig) in humans and animals. Despite reduced levels of Ig, smokers have been found to have increased levels of autoantibodies especially rheumatoid factor (Masdottir et al. 2000). In a study of RA patients, interactions between smoking and the HLA-DRB1 genotype resulted in a high risk of seropositive RA (Padyukov et al. 2004). In a study by Manthorpe et al, investigating SS patients, a lower focus score was found in smoking patients compared with non-smoking patients (Manthorpe et al. 2000). They further found reduced Ro/SSA and La/SSB autoantibodies in the peripheral blood of smoking patients (Manthorpe et al. 2000). A previous study by Tengner et al demonstrated the presence of Ro and La autoantibody-producing cells in salivary glands from patients with SS, and reported a correlation between the presence of autoantibodies in sera and the presence of autoantibody-producing cells in glandular biopsies (Tengner et al. 1998). The smoking status of the patients in the study is not known. From the findings of Tengner et al, Manthorpe proposed a hypothesis that smoking lowers the focus score by reducing the accumulation of lymphocytes in salivary glands. This leads to a lower production of Ro/SSA and La/SSB autoantibodies locally which subsequently leads to normal levels of circulating Ro/SSA and La/SSB autoantibodies. In our cohort of 125 Ro/SSA-positive patients (**Paper I**), 78 were current or former smokers. Of these 78 patients, 29% were current smokers which is a statistically significant difference compared to the percentage of adult daily smokers in Stockholm 2003 (17.8%). We also found that the majority of the smokers were lupus patients and that smokers had significantly lower levels of anti-Ro52 and anti-La compared to non-smokers (never smokers and former smokers) (**Paper II**). Our findings support previous findings on the immunosuppressive effects of cigarette smoke on the adaptive immune system. Among the 18 patients with pSS only one was a smoker which we suggested was due to the fact that mucosal dryness makes smoking less pleasurable. If one applies Manthorpe's proposed hypothesis, our finding of a single smoking SS patient might be due to the fact that the other smoking SS patients have normal levels of anti-Ro/SSA and are thus not included in our cohort of anti-Ro/SSA-positive patients.

Smoking has been associated with DLE patients (Gallego et al. 1999; Miot et al. 2005) and also with SLE and increased disease activity (Ghaussy et al. 2003). Overrepresentation of smokers in CLE has been reported and in one study as many as 82% of the CLE patients were found to be smokers (Boeckler et al. 2005). Our findings call for investigation of smoking status also in anti-Ro/SSA negative CLE patients. A

combination of smoking and complement deficiency has also been shown to be a risk factor for CLE in men (Boeckler et al. 2005). Studies have further revealed that smoking might interfere with anti-malarial treatment in CLE patients leading to a reduced response to treatment (Rahman et al. 1998; Jewell and McCauliffe 2000). One possible mechanism that has been proposed is that the metabolism of anti-malarials is inactivated by the cytochrome P-450 enzyme complex and that polycyclic aromatic hydrocarbons found in cigarette smoking are P-450 inducers (Schein 1995). Smoking cessation advice and information about the adverse effects of smoking are important and should be included in the information given to these patients.

We further investigated the presence of autoantibody-producing cells in skin specimens of SCLE lesions by immunohistochemical analyses with biotinylated antigens (**Paper II**). Skin biopsies from four SCLE patients (3 smokers and 1 non-smoker) with OD levels of Ro52 varying from 1.0 to 1.8 were stained. We found no autoantibody-producing cells, which was not unexpected, since T cells rather than B or plasma cells constitute the dermal infiltrates. In salivary glands germinal centres and plasma cells are found, in contrast to affected skin in CLE where these are only possibly found in the dense lymphocytic infiltrates of lupus profundus, an uncommon subtype of CCLE. The gene-environmental interaction between smoking and the HLA-DR genotype in seropositive RA patients, as demonstrated by Padyukov et al (Padyukov et al. 2004) might also be relevant in anti-Ro/SSA-positive CLE patients since many of these patients are smokers as demonstrated in our cohort. This remains however to be studied since HLA typing on our cohort has not been performed.

2.4.2.4 Hormones

Nearly 80% of patients with autoimmune diseases are women (Jacobson et al. 1997) indicating that sex hormones influence the immune system. The female-to-male ratio is 3-6:1 in SCLE and 3:1-2 in CCLE. Among patients with SLE in childbearing ages 90% are women but in prepubertal and postmenopausal ages the female preponderance is being lower. Studies have shown that women have an increased antibody production and estrogen has been found to increase autoantibody-production as well as proinflammatory cytokine production (e.g. TNFα, IL-1β) (Kanda et al. 1999; Whitacre 2001). Studies have also shown that changes in the concentration of estrogen, for example in pregnancy, or administration of exogenous estrogens are associated with an increased risk of developing SLE or DLE (Meier et al. 1998; Khamashta 2006). Pregnancy is associated with a shift to a Th2 immune response, and since SLE has been suggested to be a Th2-mediated disease, SLE patients tend to worsen during pregnancy in contrast to Th1 mediated diseases such as RA which tends to improve (Wilder 1998). As previously mentioned estradiol has been found to induce binding of anti-Ro/SSA to the cell surface of human keratinocytes triggering CLE (Furukawa et al. 1988; Jones 1992a; Zhang et al. 2000). Further evidence of female preponderance is shown in our finding in **Paper I** that of the 1323 Ro/SSA positively tested persons in Stockholm county 1996-2002, 85% were women. Among our study population of CLE, 22 out of 26 patients with SCLE and CCLE were women. Taken together, the evidence suggests that female hormones may contribute to the autoimmune response in a lupus susceptible individual.

2.4.2.5 *Infectious agents*

Viruses, particularly Epstein-Barr virus, cytomegalovirus and retrovirus, have been associated with the development of SLE and SS (reviewed in James et al. 2001). Induced antibodies or T cells that are stimulated in response to infectious agents may cross-react with self-antigens. This is called molecular mimicry and has been proposed as a triggering mechanism in autoimmunity. Electron microscopy studies in the 1970's revealed tubular structures in the skin of chronic discoid and SLE patients proposing viral infection to be of pathogenic importance in CLE as well (Haustein 1973). In recent times there have been no new studies in this field.

2.5 THE AUTOIMMUNE INFLAMMATION IN THE TARGET ORGAN

The distribution of skin lesions in CLE suggests that UV radiation is involved in the pathogenesis. An experimental model used for investigations of the pathogenesis of CLE in vivo in humans is the photoprovocation test. Photoprovocation studies have shown that the action spectrum of LE is within the UVB (290-320 nm) and UVA (320-400 nm) range and that the photoprovoked LE lesions can persist for months (Lehmann et al. 1990). Not only UV but also visible light (400-800 nm) has been shown to induce lesions (van Weelden et al. 1989; Kind et al. 1993). Serial biopsies can be performed from the induced skin lesion after photoprovocation to investigate immunohistochemical and micromorphological changes in early and evolving LE lesions respectively (Nyberg et al. 1999). Serial biopsies provide a unique opportunity to study the pathology and relate the clinical course of the disease to molecular events taking place in the autoimmune target organ.

UV radiation induces the release of cytokines from different cells promoting cutaneous inflammation in CLE. TNFα and IL-1 are proinflammatory cytokines that may be released from keratinocytes upon UV radiation (Kupper et al. 1987; Kock et al. 1990). UV radiation can also result in mast cell degranulation and release of their intracellular stores of TNFα into the dermis (Walsh 1995). TNFα and IL-1 in turn promote the release of many other proinflammatory cytokines (e.g. IL-6, IL-8, IFN-γ) leading to an inflammatory cell migration (reviewed in Bennion and Norris 1997). UVB also releases IL-10. The cascade of inflammatory mediators released into the epidermis and dermis by UV radiation induces the expression of adhesion molecules (ICAM-1, vascular adhesion molecule-1 (VCAM-1), E-selectin) on keratinocytes and leukocytes promoting leukocyte migration through the endothelial barrier into the skin (Bennion and Norris 1997). Upregulation of adhesion molecules in the skin after photoprovocation (Stephansson and Ros 1993; Nyberg et al. 1999) as well as in serum (Nyberg et al. 1997a) has been described in various subtypes of CLE. The activated lymphocytes and macrophages also produce cytokines in the dermis, which further enhances the migration of more leukocytes and eventually trigger cytotoxic damage to keratinocytes. Recent studies have also revealed the presence of cutaneous IFN-α producing cells in the skin of SLE and CLE patients (Blomberg et al. 2001; Farkas et al. 2001). Furthermore, local production of IFN-α has been shown to induce a Th1 biased inflammatory immune response with recruitment of T cells into the skin in CLE thus constituting an additional factor contributing to the immunoreaction in CLE (Wenzel et al. 2005b).

2.5.1 High mobility group box chromosomal protein 1

The initial events and time course of mediators of inflammation in the formation of a skin lesion in CLE is not known. The role of the novel cytokine high mobility group box chromosomal protein 1 (HMGB1) in the pathogenesis of CLE was investigated in Paper IV and V.

HMGB1 is a nuclear protein that binds to DNA and is involved in the organization of the chromatin (Goodwin et al. 1973). It is ubiquitously present in the nuclei of all cells and is a member of the HMGB family, which includes HMGB1, HMGB2 and HMGB3. In contrast to HMGB1, HMGB2 is restricted to the testis and lymphoid tissue in adults (Ronfani et al. 2001) while HMGB3 expression has only been reported during embryogenesis (Vaccari et al. 1998). The abbreviation HMG refers to the rapid electrophoretic migration pattern seen in polyacrylamide gels. Beside HMGB there are also HMGA and HMGN, which all have the capacity to bind to DNA.

2.5.1.1 Extranuclear functions of HMGB1

HMGB1 also has extranuclear functions. HMGB1 is actively secreted by stimulated monocytes/macrophages (Wang et al. 1999) or passively released by necrotic or damaged cells (Scaffidi et al. 2002). A recent study proposed that HMGB1 may be released during apoptosis as well (Bell et al. 2006). Qin et al showed that the exposure of apoptotic cells to macrophage cultures stimulates the release of HMGB1 in a dose dependent matter (Qin et al. 2006). During its secretion, HMGB1 exits the nucleus, is transported through the cytoplasm and is actively released to the extracellular space (Gardella et al. 2002). The receptor for advanced glycation end products (RAGE) is the most functional receptor for HMGB1 (Kokkola et al. 2005). By the binding of HMGB1 to RAGE, HMGB1 can exert its functions. The RAGE receptor is also expressed in the skin and may thus act via this pathway to carry out its functions (Lohwasser et al. 2006). HMGB1 has recently been found to act as a proinflammatory cytokine when secreted (Andersson et al. 2002) in both acute and chronic inflammatory conditions such as septic chock, RA and myositis (Wang et al. 1999; Kokkola et al. 2002; Ulfgren et al. 2004). It stimulates the synthesis of TNFα and IL-1β in human PBMCs and thereby promote chronic inflammation (Andersson et al. 2000). TNFα and IL-1β may on the other hand induce HMGB1 (Wang et al. 1999) and together the three cytokines form a proinflammatory loop, since they can induce the release of each other. HMGB1 has also been reported to activate dendritic cells, and thereby act as an endogenous immune adjuvant (Rovere-Querini et al. 2004). HMGB1 was also recently shown to alter the subcellular distribution of autoantigens, making the autoantigen accessible for immune responses (Sanford et al. 2005). HMGB1 could hypothetically thus enhance the cell surface expression of Ro/SSA in CLE patients as well, but further investigation is needed to verify this hypothesis.

2.5.1.2 HMGB1 in CLE

The expression of HMGB1, TNFα and IL-1β in spontaneously occurring skin lesions of CLE was investigated by immunohistochemistry (**Paper IV**). An increased expression

of HMGB1 in the epidermis ($p<0.01$) and in the dermal infiltrates ($p<0.001$) of CLE lesions was observed in comparison with unaffected skin. Translocation to the cytoplasm as well as secretion of HMGB1 was also found. We propose that the extracellular staining indicate either release from activated inflammatory cells, or that the keratinocytes constitute a novel source contributing to the extracellular pool of HMGB1. The latter possibility is supported by a recent finding of Bell et al, who found that HMGB1 can also be released during apoptosis (Bell et al. 2006) and the fact that keratinocytic apoptosis is increased in lupus. Furthermore, we proposed that while UV radiation initiates the formation of the lesions, HMGB1 may be of importance in sustaining inflammation.

We also found an increased expression of TNFα and IL-1β in CLE. To our knowledge, this is the first investigation describing TNFα in the skin of CLE patients. As a consequence, the increased amount of TNFα and IL-1β observed in CLE leads to a higher expression of HMGB1 as previously described. To understand at what stage and in what sequential order each cytokine is expressed, the sequence of events in HMGB1, TNFα and IL-1β expression was investigated (**Paper V**). Serial biopsies from evolving experimentally photoinduced cutaneous lesions in patients with CLE were stained with immunohistochemistry for HMGB1, TNFα and IL-1β. The highest expression and extracellular deposition of HMGB1 corresponded to the clinically most active phase of photoinduced lesions of CLE. At the same time only low levels of TNFα and IL-1β were observed, whereas the expression of these cytokines tended to peak in the late fading lesions. We propose that HMGB1 is an early mediator of inflammation and that it may contribute to the upregulation of TNFα and IL-1β in UV induced CLE. The lower expression of TNFα and IL-1β compared with the spontaneously occurring lesions in Paper IV could reflect, either a difference in nature between induced and spontaneously occurring lesions, or may indicate that HMGB1 is indeed an early factor in the development of the lesion.

In these immunohistochemical analyses we primarily used monoclonal antibodies to reduce cross-reactivity. We used a semiquantitative method to analyze the cytokine expression focusing on the intra-/extracellular localization of HMGB1. Computerized image analysis of the staining was not performed, since we are not aware of a reliable method to determine localization and intra-/extracellular distribution of staining. In our opinion, such evaluation requires manual scoring. In our experience, manual evaluation of more traditional tissue stainings performed blinded and independently by several observers yields results comparable to computerized image analysis (Cunnane et al. 1999).

In 2006 Bell et al (Bell et al. 2006) showed that HMGB1 can be secreted from apoptotic cells, which contradicts our statements that apoptotic cells are poor HMGB1 secreters in the Introduction and Discussion sections of Paper IV and V. A methodological weakness of Paper V is that it is not feasible to study large numbers of samples for practical and ethical reasons, since it is not possible to obtain enough biopsies from a large group of patients.

2.6 THE Ro ANTIGENS-, CELLULAR FUNCTION OF Ro52 AND ITS RELATION TO AUTOIMMUNITY

2.6.1 The Ro60 antigen

Presence of the Ro60 antigen is important for cell survival after UV irradiation (Chen et al. 2003). Mice lacking the Ro60 protein develop a lupus-like syndrome, thus Ro60 may not just be a target but may also be important for the prevention of autoimmune disease (Xue et al. 2003). It was also shown that mice lacking Ro60 were photosensitive with increased numbers of apoptotic keratinocytes i.e sunburn cells, suggesting that loss of Ro60 function could contribute to photosensitivity (Xue et al. 2003). It was further suggested that entry of antibodies into keratinocytes could potentially enhance sensitivity to UV radiation resulting in increased apoptosis of keratinocytes (Xue et al. 2003).

2.6.2 The Ro52 antigen and proposed function

The Ro52 protein belongs to a family of tripartite motif proteins (TRIM) (Reymond et al. 2001), and is also known as TRIM 21. TRIM proteins share the same domain structure with a RING-finger, a B-box, and a coiled-coil, and have been shown to participate in the polyubiquitination pathway. Ubiquitin (Ub) which is a highly conserved protein, tags intracellular proteins for enzymatic degradation. Ubiquitin is activated by the enzyme E1 which is then transferred to E2. The Ub ligase E3 catalyzes the transfer of Ub to a specific target protein. Multiple Ubs are added, and the polyubiquitinated proteins are degraded by a protease complex (proteasome). The ubiquitination pathway is illustrated in Figure 5.

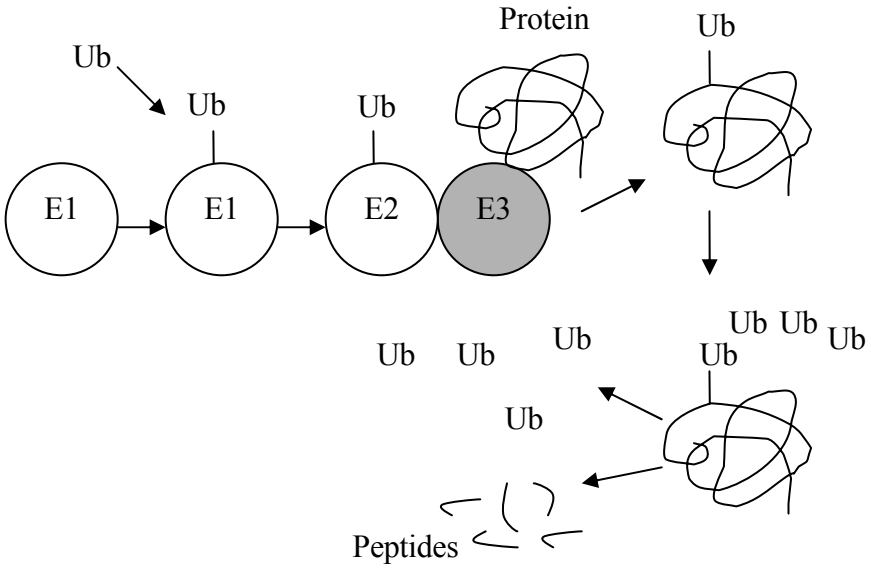

Figure 5. Ubiquitination pathway.

Many of the TRIM proteins have recently been identified as E3 ligases in the ubiquitination pathway, and act as intracellular regulators of processes such as apoptosis, proliferation and signal transduction (Dho and Kwon 2003; Horn et al. 2004).

The function of Ro52 has remained completely unknown for a long time. In an attempt to define the function and the expression pattern of Ro52, PBMCs from Ro/SSA-positive patients with pSS (n=20) and SLE (n=18) were collected (**Paper VI**). Real time PCR revealed that Ro52 expression in PBMCs is increased in patients compared with healthy controls (n=18). No difference was seen between the pSS and SLE groups. B cells showed the highest Ro52 mRNA expression particularly in naive B cells. The study further showed that Ro52 was mainly localized to the cytoplasm but a minor nuclear presence was also observed. In vivo and in vitro ubiquitination assays showed that Ro52 is an E3 ligase which is dependent on its RING finger domain. In the same study B cells were also transfected with Ro52 which lead to decreased proliferation. Over-expression of Ro52 was also shown to induce apoptosis in a dose dependent manner. Taken together the results from Paper VI suggest that the high expression of Ro52 in pSS and SLE patients suppresses cell proliferation and increases apoptosis.

It has been demonstrated that patients with CLE show higher count of epidermal apoptotic nuclei after UV irradiation (Kuhn et al. 2006) as well as decreased proliferation of fibroblasts (Nyberg et al. 2000). Furthermore, Ro/SSA-positive CLE show a tendency toward increased accumulation of apoptotic cells compared to Ro/SSA-negative CLE (Kuhn et al. 2006) and a higher apoptotic rate has been shown in SCLE compared with CCLE (Baima and Sticherling 2001). In one study, expression of Ro and La antigens were found to be increased in the skin of patients with photosensitive forms of LE, such as SCLE compared with patients with non-photosensitive forms of LE and normal subjects (Ioannides et al. 2000). In the same study, patients who were high antigen expressors were more likely to have circulating Ro/SSA autoantibodies than those who were low antigen expressors. Monoclonal antibodies against Ro may help to clarify the role of the Ro antigen in the skin of CLE patients.

Hypothetically, the higher Ro/SSA antigen expression seen in SCLE compared to less photosensitive LE, for example CCLE, may lead to a higher E3 ligase activity of Ro52 in SCLE, promoting a higher apoptotic rate. In skin with a high expression of Ro52, the E3 ligase activity promoting apoptosis may be an initial step in the pathogenesis of CLE. Thus, the Ro52 antigen functions as an E3 ligase and promotes apoptosis, and may constitute a molecular basis for the pathogenesis of CLE in Ro/SSA-positive patients.

2.7 PROGNOSIS IN RELATION TO Ro/SSA ANTIBODIES

Data from the original study cohort by Sontheimer et al suggested that SCLE is a subset with intermediate severity, falling between CCLE and SLE (Sontheimer et al. 1979). The prognosis of CLE is regarded as more favorable than that of SLE. A ten year follow-up study by Sontheimer et al showed a relatively stable clinical course with little

progression or mortality (Sontheimer 1989). However, as previously mentioned, 50% of SCLE patients and 5-10% of DLE patients develop SLE, therefore the physician should routinely evaluate CLE patients for the presence of other autoimmune diseases which may develop. In CLE, the prognostic value of the ACR criteria has been debated (reviewed in Albrecht et al. 2004). From a dermatological point of view the ACR criteria are too sensitive and include four parts based on mucocutaneous manifestations, see Table 1. A patient with CLE can thus fulfil four criteria for SLE diagnosis based solely on skin and mucosal manifestations. The ACR criteria in their present version, do not support the clinician in evaluating prognostic factors in patients with different forms of CLE.

We addressed whether the fine specificity of anti-Ro/SSA, namely anti-Ro52 and anti-Ro60, may serve as a prognostic tool to reveal patients predisposed to developing systemic disease (**Paper II**). We used ELISA to describe the serological characteristics in different clinical presentations of patients with Ro/SSA autoantibodies. The study included 96 patients, all of whom participated in the epidemiological study (Paper I). All patients had self-reported photosensitivity and skin symptoms. SCLE patients with a diagnosis of systemic disease such as SLE, SS or RA showed higher levels of anti-Ro52 ($p<0.05$) and anti-La ($p<0.05$) than SCLE patients with manifestations confined to the skin. Taken together the results of this paper demonstrate that ELISA testing of fine specificities of Ro/SSA autoantibodies could be used as a prognostic tool since patients with SCLE with high levels of anti-Ro/SSA and anti-La/SSB, may reveal a subset of patients with a higher risk of developing systemic manifestations. ELISA testing could thus serve as a complement to the ACR criteria in distinguishing between CLE patients with or without SLE.

Prognostic markers for CLE are less well known than the risk factors that trigger the onset of the disease. In addition to the findings in Paper II, a previous study has also shown that CLE patients with signs of nephropathy (proteinuria, hematuria), arthralgia and presence of high titers of ANA are at risk of developing SLE (Tebbe et al. 1997). Furthermore, a ten year retrospective follow-up study reported that anti-Ro/SSA-positive patients have a dynamic disease process over time, and that disease progression occurs in the majority of Ro/SSA-carrying patients (Simmons-O'Brien et al. 1995). Less is known about disease development in a shorter-term perspective, although late onset of SCLE and SLE has been reported to have a more benign disease course with less serious organ involvement (Ward et al. 1995; Ho et al. 1998).

We conducted a prospective study of clinical outcome in 102 patients with Ro/SSA autoantibodies in their serum at baseline 2003 (**Paper III**). We re-examined the patients two years after the initial study, and found that 20% of the patients had dermatological or rheumatological disease progression with the development of drug-induced SCLE (n=2), DLE (n=1), polyarteritis nodosa (n=1), RA (n=1), SS (n=3), SLE (n=2), vasculitis (n=2), squamous cell carcinoma (n=1), psoriasis arthritis (n=1) and both systemic and cutaneous relapses of LE (n=9). Patients with disease progression had a mean age of 53 years compared to 59 years in the entire cohort. Eighteen of the twenty patients with disease progression were women. One patient with SCLE and SLE developed SS during the observation period. As with other patients with SS, clinicians should be aware of the potential risk of development of B cell lymphoma in these

patients. One patient developed squamous cell carcinoma at the site of a DLE lesion. The risk of developing malignancy in DLE has previously been described (Millard and Barker 1978) and should be taken into account by all specialities involved in the treatment of SLE patients with DLE manifestations. Furthermore, low levels of anti-Ro/SSA and anti-La/SSB at baseline still correlated with disease limited to the skin. Patients also frequently self-reported arthralgia. This underreported symptom has been found to affect the quality of life in psoriatic patients (Zachariae et al. 2002), and may also affect the quality of life in the patients of this study.

The data from Paper III thus confirm the observation that anti-Ro/SSA-positive patients have a dynamic disease process, which was also evident in the short-term perspective of two years. There are however, some limitations to this study. The short follow-up time in a heterogenous group of patients is not sufficient to make general statements about prognosis. A further limitation with the study is that after evaluation of the questionnaire responders, only the 31 patients with "skin problems" were clinically investigated, and not all the patients. The aim of the questionnaire was to identify patients with new or changed skin symptoms, who were therefore at risk of developing CLE.
Another critique of the study is that we did not choose to retest Ro/SSA autoantibodies to investigate possible serological changes compared to the baseline in 2003. Previous serial measurements over a two-year time period, of Ro/SSA autoantibodies in sera from patients with SLE and SS, have however shown that fluctuations occur during the course of the illness but they only have limited clinical value in predicting activity or exacerbations of the disease (Praprotnik et al. 1999). The fine specificity of Ro/SSA autoantibodies in the same study was also found to remain stable during the two-year time period (Praprotnik et al. 1999).

In conclusion, the physician must be aware of the manifestations that anti-Ro/SSA-positive patients are at risk of developing, and regular clinical appointments should be emphasized.

3 CONCLUDING REMARKS

The aims of this thesis were to determine the incidence and prevalence of SCLE as well as to characterize patients with Ro/SSA autoantibodies. Furthermore, we aimed to investigate and increase our knowledge of the pathogenesis of CLE and of the function of the autoantigen Ro52. This design has given us the opportunity to gain an increase in our understanding of the clinical aspects, as well as of some of the molecular aspects of the Ro/SSA-positive patients studied.

By making use of the knowledge that the majority of patients with SCLE are positive for Ro/SSA autoantibodies and by the unique Swedish registry-based opportunities we identified all Ro/SSA-positive patients in Stockholm diagnosed over a seven year time period. Through this cohort we identified SCLE patients and were able to determine the incidence and prevalence of SCLE for the first time. The estimated incidence of Ro/SSA-positive SCLE in Stockholm is 0.7/100,000 per year and the prevalence is 6.2-14/100,000. SCLE is thus a rare subset of lupus, and the lack of knowledge about the number of affected patients makes this disease even more invisible. However, our data together with prevalence estimations for CCLE demonstrate that CLE is probably a more prevalent disease than SLE. Our findings should be helpful to support not only the SCLE patients but the whole CLE patient group in being acknowledged, and form a basis for planning of health care resources. The results also provide the first opportunity to assess future health-economic considerations in order to motivate the use of expensive biological therapies. We further conducted a prospective study on our cohort of Ro/SSA-positive patients to evaluate disease progression in a short-term perspective of two years. To the best of our knowledge, no prospective studies concerning the clinical outcome in patients with Ro/SSA autoantibodies have been reported. These patients, showing a heterogenous clinical spectrum, had an unexpectedly dynamic disase process. Our results demonstrate many of the manifestations Ro/SSA-positive patients are at risk of developing and underline the importance of regular clinical follow-up in cooperation between dermatologists and rheumatologists.

CLE patients can develop SLE but prognostic factors are not well defined. To explore the differences in serological findings in different diagnostic groups, we analyzed the levels of antibody directed against the different epitopes of Ro/SSA and La/SSB with ELISA. Findings of higher autoantibody levels in CLE with systemic manifestations compared to patients with only cutaneous manifestations indicate that quantification of the fine specificites of anti-Ro/SSA and anti-La/SSB may be of value as a prognostic tool which could serve as a complement to the ACR criteria in photosensitive cutaneous diseases. We further showed that smokers had lower levels of autoantibodies suggesting that interaction between the immune system, genes and environmental factors contributes to the development of CLE.

Our studies demonstrate the expression of TNFα in CLE lesions for the first time. We have also investigated the expression of the cytokine HMGB1 in spontaneously occurring and experimentally induced CLE lesions, in order to explore its role in the pathogenesis. Our findings of increased HMGB1 in CLE suggest that HMGB1 has a

role in the cytokine cascade and constitutes a potential future therapeutic target, particularly for patients with refractory disease. It would also be interesting to investigate the expression pattern in other dermatological diseases, such as psoriasis, lichen planus and mucosis fungoides.

Anti-Ro/SSA screening is often performed in clinical practice, but why the Ro52 antigens are targeted by the autoantibodies and the function of Ro52 is not known. We demonstrated that Ro52 is overexpressed in PBMCs of patients with pSS and SLE and that Ro52 is a RING-dependent E3 ligase involved in ubiquitination. Further results show that overexpression of Ro52 in a mouse B cell line leads to decreased growth and increased apoptotic cell death after activation, indicating that Ro52 plays a role in cell cycle regulation and/or apoptosis.

A proposed hypothesis for the pathogenesis of Ro/SSA-positive SCLE based on the findings in the present thesis and the observations of others are summarized below and also illustrated in Figure 6. Ro/SSA-positive SCLE patients can display systemic and non-systemic manifestations. We found that if SCLE patients display systemic manifestations they have higher levels of anti-Ro52 antibodies (Paper II). Previous studies have shown that high anti-Ro52 antibody levels correlate to high Ro52 antigenic levels in keratinocytes (Ioannides et al. 2000). Our study reveals that Ro52 is an E3 ligase that regulates cell death and that over-expression of Ro52 mediates apoptosis in a dose-dependent manner in a mouse B cell line (Paper VI). If this also is true for SCLE patients, then high expressors of Ro52 such as SCLE patients with SLE, may have a higher number of apoptotic keratinocytes than SCLE patients without SLE (low expressors). Furthermore, the cytokine HMGB1 was found to be over-expressed in SCLE lesions (Paper IV). HMGB1 has recently been found to be released during apoptosis (Bell et al. 2006) and we propose that the apoptotic keratinocytes release HMGB1. HMGB1 together with TNFα and IL-1β, which were also expressed in SCLE lesions, form a proinflammatory loop which promotes inflammation. We propose that while UV radiation initiates formation of the lesions, HMGB1 may be of importance in sustaining inflammation.

In the future, it would be interesting to compare our findings with those described in other autoimmune diseases. A base has been formed for the continued long-term follow-up of the Ro/SSA-positive patients for the study of disease development and to verify or modify our epidemiological results. The database we have built may also be used for other epidemiological projects which will result in increased knowledge of for example the incidence of malignancy in these patients. Further laboratory experiments of keratinocytic cell cultures of CLE lesions and the use of monoclonal antibodies against HMGB1 and Ro52 will increase our knowledge of the role of these proteins in apoptosis. An additional line of future research would be to perform experiments to investigate the role of the RAGE receptor in the skin and to transfect keratinocytes with Ro52. It is important to reveal the mechanisms of the autoimmune inflammation of these diseases to understand the pathogenesis, but also to identify potential therapeutic targets.

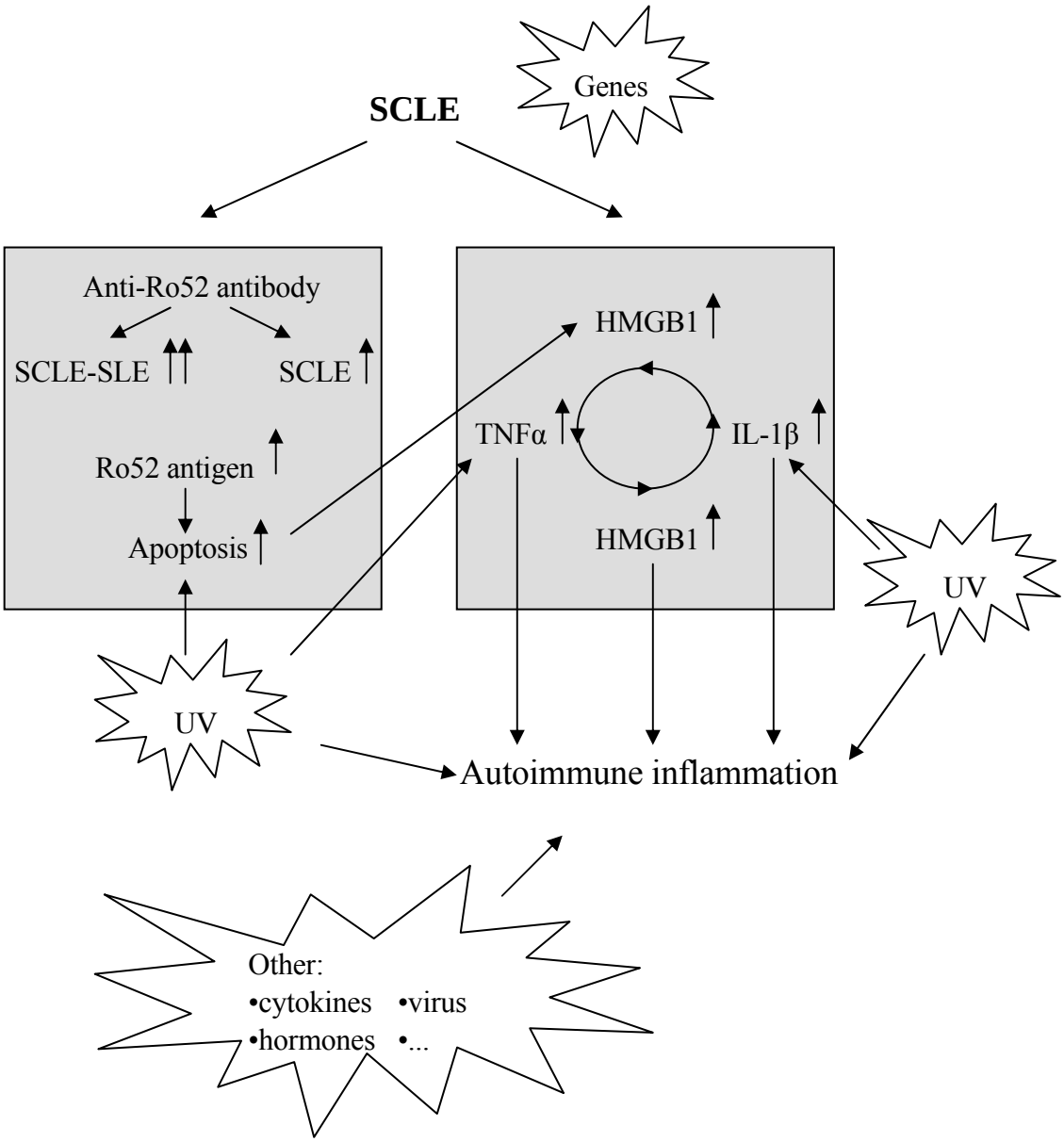

Figure 6. A summary of the hypothetical mechanisms in the pathogenesis of Ro/SSA positive SCLE.

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