Mixed Connective Tissue Disease, Myositis and Systemic Lupus Erythematosus

Immunological and genetic studies in three related rheumatic autoimmune diseases

By

Adla Bakri Hassan

Stockholm 2002
To Diab and Mohamed

To my parents, sisters
& brothers
"In the name of Allah,
Most Gracious, Most Merciful"
"Of knowledge it is only a little that is
communicated to you, (O men!)"
ABSTRACT

Mixed connective tissue disease (MCTD), polymyositis (PM)/dermatomyositis (DM) and systemic lupus erythematosus (SLE) are chronic, rheumatic, systemic inflammatory disorders. The disorders depend on several factors of both genetic and environmental origin. They are heterogeneous diseases but all are characterized by the presence of various autoantibodies directed to nuclear and/or cytoplasmic components. Neither the pathogenic role nor the mechanisms driving the autoantibody production in these diseases are fully understood.

The main objective of my thesis was to get an increased knowledge of the mechanisms driving autoantibody production and their importance in disease mechanisms. Therefore, I studied the relation of autoantibody production to disease activity and furthermore to serum levels of cytokines and also to certain genetic markers that could be of interest in these aspects in three different but related autoimmune rheumatic diseases such as MCTD, PM/DM and SLE.

The anti-U1snRNP (68 kDa, RNP-A and RNP-C) autoantibodies in MCTD patients and the anti-Ro and anti-La autoantibodies in SLE patients had a coordinated expression indicating that these groups of autoantibodies are driven by a similar mechanism in the same disease. Nevertheless, the anti-U1 RNP autoantibodies levels were highly fluctuating with time in MCTD patients, while anti-Ro and anti-La autoantibodies levels in SLE patients were fixed early in the disease and hardly changed with time indicating that autoantibodies are derived and maintained by different mechanisms in different diseases. Correlations between autoantibodies and overall disease activity or specific clinical features in different diseases were observed. Increased serum levels of the cytokines TNF-α and IL-10 were demonstrated in patients with PM/DM and MCTD compared to controls, but not in SLE. There were no correlations between individual cytokine levels and disease activity in any of the investigated disorders. In MCTD patients correlations between anti-U1 snRNP antibody levels and cytokines levels particularly for TNF-α were demonstrated. A high TNF-α/IL-10 ratio was associated with presence of anti-Jo-1 autoantibodies in PM/DM patients. This ratios seemed to have a genetic basis. An association between certain MHC and non-MHC genes and development of disease, cytokine production and presence of autoantibodies in patients with MCTD and PM/DM has been determined. In particular TNF2 allele of the -308TNFA gene was associated with PM/DM diagnoses.

In conclusion, these immunological studies in MCTD, SLE and PM/DM provided information about immunological factors and some mechanisms that could drive these factors in these diseases. The diverse autoantibody patterns in MCTD and SLE could indicate that different mechanisms drive and maintain various autoantibodies, while the co-ordinated expression indicates that autoantibodies within one disorder could be driven by the same mechanism. The current studies imply that upregulation of cytokine production is likely to be part of the mechanisms underlying autoantibody production. Variations in cytokines and autoantibody production may have a genetic basis. The genetic studies suggest that the HLA-DRB1*04 and -DRB1*03 positive haplotypes rather than the separate markers in the HLA region are important factors in the disease susceptibility for MCTD and PM/DM, respectively. Finally, these genetic studies were the first to enlighten the role of cytokine gene polymorphisms and also to provide the basis for haplotype analysis in patients with MCTD and adult PM/DM.

**Key words:** MCTD, SLE, PM/DM, autoantibodies, cytokines, MHC, polymorphisms.
PREFACE

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


V. **Adla B. Hassan**, Liene Nikitina-Zake, Carani B. Sanjeevi, Ingrid E. Lundberg and Leonid Padyukov. Proinflammatory haplotype (MICA5.1/ TNF2/ TNFa2/DRB1*03) is associated with poly- and dermatomyositis. Submitted manuscript to Arthritis and Rheum.


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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AuAb(s)</td>
<td>Autoantibody (s)</td>
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<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CTDs</td>
<td>Connective tissue diseases</td>
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<tr>
<td>DM</td>
<td>Dermatomyositis</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigens</td>
</tr>
<tr>
<td>IIM</td>
<td>Idiopathic inflammatory myopathy</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
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<tr>
<td>MCTD</td>
<td>Mixed connective tissue disease</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MIC</td>
<td>MHC class I-related chain gene</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PM</td>
<td>Polymyositis</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>SLAM</td>
<td>Systemic lupus activity measure</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SS</td>
<td>Sjögren’s syndrome</td>
</tr>
<tr>
<td>SSc</td>
<td>Systemic sclerosis</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNFA</td>
<td>Tumor necrosis factor-alpha gene</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumor necrosis factor-alpha gene microsatellite</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha molecule</td>
</tr>
<tr>
<td>U1snRNP</td>
<td>Uridylicate small nuclear ribonucleoprotein</td>
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</table>
INTRODUCTION

Autoimmune rheumatic diseases are disorders that result from breakdown of self-tolerance and/or as a consequence of an abnormal immune response (autoimmune response). Neither the initiating factors nor the maintaining factors for the autoimmune response are well understood. However, several factors of both genetic and environmental origin have been implicated (Cooper et al. 1999). Presence of autoantibodies (AuAbs) is one of the characteristic features of many autoimmune diseases, although the specificity of these AuAbs varies between different clinical phenotypes. An increased understanding of the mechanisms which are involved in AuAb production and their relation to disease activity and clinical symptoms is likely to improve our current knowledge of the mechanisms that cause these disorders, and thereby improve our possibility to develop new therapies for these conditions.

Autoantibodies

Autoantibodies are antibodies that may react with a wide range of self-antigens of nuclear, cytoplasmic, or tissue origin (Tan 1989). The role of these AuAbs (i.e. if they are pathogenic) in many of the autoimmune diseases is still not clear. From a clinical aspect AuAbs serve as diagnostic and in some instances as prognostic markers. In autoimmune diseases AuAbs are mainly of IgG isotypes such as antibodies to U1 RNP in mixed connective tissue disease (MCTD) and systemic lupus erythematosus (SLE). However, AuAbs of IgM isotype are often present. AuAbs that recognize the Fc portion of IgG are termed rheumatoid factors (RF) and are present in most rheumatoid arthritis (RA) patients, in some MCTD patients and rarely in SLE patients (Table 1). Whether RF contributes to the disease process in RA is still not understood, but current concepts include a role in facilitating antigen presentation (Carson et al. 1991).

The importance of AuAbs in the pathogenesis of different autoimmune diseases is suggested, since IgG is a major component of immune complexes and may contribute to disease activity by activating complement and stimulating cytokine synthesis. Moreover, the potential of immune complexes to contribute to tissue damage indicates a potential pathogenic role of these AuAbs, but whether these AuAbs exert their effects directly and cause tissue injury or indirectly through immune complexes is still unclear.
Several types of AuAbs are present in sera of patients with different autoimmune rheumatic diseases (Table 1). It has been reported that AuAbs that recognize soluble RNA proteins such as Ro/SSA, La/SSB, Sm and U1 RNP mostly occur in association with each other, but certain AuAb profiles are associated with different clinical manifestations. In this thesis I have focused on the importance of anti-U1 RNP, anti-La, anti-La and anti-Jo-1 AuAbs, which are characteristic for each of MCTD, SLE and polymyositis/dermatomyositis (PM/DM), respectively.

Table 1. Autoantibodies in different autoimmune rheumatic diseases

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>MCTD</th>
<th>PM</th>
<th>SLE</th>
<th>RA</th>
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<tbody>
<tr>
<td>U1 RNP</td>
<td>&gt;95%</td>
<td>0%</td>
<td>20-40%</td>
<td>-</td>
</tr>
<tr>
<td>Sm</td>
<td>Rare</td>
<td>Rare</td>
<td>0-30%</td>
<td>Rare</td>
</tr>
<tr>
<td>Ro/SSA</td>
<td>33%</td>
<td>0-18%</td>
<td>12-69%</td>
<td>10-33%</td>
</tr>
<tr>
<td>Ro/SSB</td>
<td>4-13%</td>
<td>0-18%</td>
<td>19-45%</td>
<td>0-22%</td>
</tr>
<tr>
<td>Jo-1</td>
<td>Rare</td>
<td>0-20%</td>
<td>Rare</td>
<td>Rare</td>
</tr>
<tr>
<td>ANA</td>
<td>80%</td>
<td>40-80%</td>
<td>&gt;90%</td>
<td>30%</td>
</tr>
<tr>
<td>DNA</td>
<td>7%</td>
<td>-</td>
<td>60-80%</td>
<td>-</td>
</tr>
<tr>
<td>RF</td>
<td>80%</td>
<td>20%</td>
<td>0-25%</td>
<td>70-85%</td>
</tr>
</tbody>
</table>


**Anti-U1-snRNP-Sm complex AuAbs**

Among the many extractable nuclear antigens (ENA) two non-histone antigens, U1 RNP and Sm, are closely associated in a multimolecular complex (Lerner et al. 1979) (Figure 1). The nuclear ribonucleoprotein (nRNP) antigens were first identified as antigens reacting with antibodies in sera of patients suffering from Sjögren’s syndrome (SS) (Anderson et al. 1961) and later they were also identified in patients with MCTD.
and SLE (Sharp et al. 1972). U1 RNP is the most abundant ribonucleic protein particle (RNP) in the nucleus, with a function in the splicing process. It consists of one small uridylate-rich RNA (U1 RNA) complexed with at least nine polypeptides (Hinterberger et al. 1983; Pettersson et al. 1984). Three of these polypeptides, named RNP 70 (or 68 kDa), RNP-A and RNP-C, are unique to U1 RNP (Figure 1). The Sm antigen-antibody system was first identified in patients with SLE (Tan et al. 1966). It was defined as a ribonuclease-resistant nuclear RNA protein precipitated with sera of SLE patients (Tan et al. 1966). The Sm antigen consists of a complex of U-rich RNA, U1, U2, U4/U6 and U5 with core polypeptides B’, B, D, D2, D3, E, F and G (Pettersson et al. 1984). Anti-U1 RNP antibodies in high titres are characteristic of MCTD while anti-Sm antibodies are specific for SLE. It was also revealed that sera from MCTD patients recognized a different major epitope on the RNP-A polypeptide molecule, when compared with sera from RA and SLE (Barakat et al. 1991).

At present there is no evidence to indicate what might cause the AuAb production in patients with MCTD or whether these AuAbs have a pathogenic relevance. In an early single study, free serum RNP tended to appear in some patients with MCTD when anti-RNP antibody titers fell or when prednisone treatment was initiated or its dose increased. It was also evident that although antibodies to RNP seemed to be stable during the course of MCTD, their appearance could be related to the presence of free serum RNP (Fishbein et al. 1978). Another study suggested that there were some fluctuations of U1 RNP AuAb levels and that they decreased with time (Pettersson et al. 1986), but whether these AuAbs fluctuated with disease activity is not known.

The prognostic relevance of the anti-U1 RNP antibodies is still not well understood. Many studies aimed to define whether the presence of anti-U1 RNP antibodies could predict the likelihood of any particular clinical characteristic or diagnosis. The ratio of anti-U1 RNP/anti-Sm and the frequency of antibodies to 70 kDa (68 kDa) protein in SLE patients were found to relate directly to the frequency of Raynaud’s phenomenon and inversely to the frequency of nephritis (Reichlin et al. 1991). This confirms an earlier study in which patients with anti-Sm antibodies were more likely to have renal disease, antibodies to double stranded DNA (dsDNA) and single stranded DNA (ssDNA) than were patients with anti-RNP antibodies (Munves et al. 1983).
Anti-Ro/SSA and anti-La/SSB AuAbs

AuAbs to two distinct cellular antigens termed Ro/SSA and La/SSB were first described in patients with SLE (Clark et al. 1969; Mattioli et al. 1974) and also later in patients with SS. The Ro/SSA and La/SSB antigens are soluble, non-histone, heterogeneous ribonucleoprotein complexes associated with small cytoplasmic RNAs (hY-RNAs) (Wolin et al. 1984). The La antigen contains one 48 kDa protein, while the anti-Ro response can target two immunologically distinct proteins, Ro60 kDa (Wolin et al. 1984) and Ro52 kDa (Ben-Chetrit et al. 1988).

The function of Ro/SSA proteins remains largely unknown, while for the La/SSB protein several functions have been proposed, such as the initiation of the translation of mRNA (Meerovitch et al. 1993). The mammalian La protein, which appears to be required for accurate and efficient RNA polymerase III transcription, has been implicated as a transcription termination factor (Gottlieb et al. 1989; Gottlieb et al. 1989). Several studies have investigated the role of anti-Ro/SSA AuAbs in the pathogenesis of SLE. The role of anti-Ro/SSA in the pathogenesis of the skin disease of subacute cutaneous lupus erythematosus (SCLE) has been suggested, since 62% of SCLE patients have antibodies to Ro particles (Sontheimer et al. 1982). Moreover, all mothers giving birth to children suffering from skin disease of neonatal lupus erythematosus (NLE) have anti-Ro/SSA antibodies and about 50% have anti-La/SSB antibodies. Other studies have been
performed implicating anti-Ro60/SSA and/or anti-Ro52/SSA AuAbs in the pathogenesis of heart block in NLE, but not anti-La/SSB antibodies (Boutjdir et al. 1997; Yukiko 2000). Whether serum levels of anti-Ro52, -Ro60 and -La AuAbs correlate with disease activity is less certain. In a previous study a temporal correlation of the levels of antibody to Ro 52, Ro 60 and La antigens was determined. Furthermore, a co-variation of AuAb levels and changes in disease activity was apparent in four SLE patients who were young with early disease onset (Wahren et al. 1998). This observation needs to be confirmed in a larger patient cohort.

**Anti-Jo-1 AuAbs**

The most commonly encountered myositis-specific AuAbs (MSAs) in the sera of myositis patients are anti-Jo-1 AuAbs (anti-histidyl-tRNA synthetase), which occur in approximately 20% of patients with PM/DM (Love et al. 1991; Targoff 2000; Hengstman et al. 2001). Presence of anti-Jo-1 antibodies is associated with a clinical syndrome (anti-synthetase syndrome) marked by myositis, interstitial lung disease (ILD), arthritis, and Raynaud's phenomenon (Targoff et al. 1989; Targoff 1992; Targoff et al. 1992; Friedman et al. 1996).

The mechanisms that drive AuAb production are still not well understood. From earlier studies it is obvious that certain AuAbs are associated with specific clinical features, but how this association is regulated is not known. Associations between MHC genes and certain AuAbs in different diseases have been reported supporting a genetic influence of AuAb profile and clinical phenotype. More recent investigations implicate that cytokine production, such as interleukin (IL)-10 and IL-6, has a role in AuAb production (Cross et al. 1999), and that this cytokine production could be influenced by both genetic and environmental factors (Gunnarsson et al. 2000).

**Cytokines**

Cytokines are important molecules in the immune response and particularly in chronic inflammation. They are low-molecular weight proteins playing a key role in cell-cell communication. They can act locally both in an autocrine and paracrine fashion or at distance through endocrine pathways. Cytokines act through specific cytokine receptors
(Benton 1991) which are either constitutively expressed or upregulated following activation. Cytokines are often classified on the basis of their preferential effect on cell mediated and humoral responses, irrespective of the producing cells, into type 1 (also named Th1 or proinflammatory) and type 2 cytokines (also named Th2 or anti-inflammatory) (Clerici et al. 1994). Type 1 cytokines (IL-2, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α)) mediate macrophage activation and delayed type hypersensitivity reactions (figure 2) (Murray et al. 1985; Cher et al. 1987; Lynch et al. 1993). Type 2 cytokines (IL-4, IL-5, IL-10 and IL-13) are important in the regulation of humoral immunity, certain types of antibody responses and allergic diseases (figure 2) (Mosmann et al. 1989; Mosmann 1991).

**Figure 2. Type 1 (Th1) and type 2 (Th2) cytokines.**

IC= Intracellular, EC=Extracellular, Mφ=Macrophage cell, NK=Natural killer cell, DC=Dendritic cells, LT-α, CRP=C-reactive protein.
Cytokines exert a multitude of different biological effects on the growth and differentiation of many cell types. The effect of a single cytokine varies depending on the target cell, concentration and when during an immune response it is released. For example, the effect of transforming growth factor (TGF)-β on the target cell depends on the cell type, its state of differentiation, activation and proliferation as well as on the total milieu of cytokines present. As an example, exposure of naïve CD4+ cells to TGF-β during their priming phase directs differentiation toward a Th1 phenotype, with the primary result being an increase in INF-γ producing cells and a decrease in IL-4-producing cells (Swain et al. 1991).

Most of the cytokines are believed to be strong cellular regulators because they suppress or induce the expression of other cytokines through synergistic and antagonistic interactions and thus suppress or promote certain diseases (Balkwill 1989; Elias et al. 1992). Type 1 and type 2 cytokines reciprocally regulate the production of each other (Liblau et al. 1995). Thus, type 2 cytokines down-regulate type 1 cytokines through the production of IL-4 and IL-10 (Gautam et al. 1992; de Vries 1995), while type 1 cytokines down-regulate type 2 cytokines through the production of IFN-γ (Figure 2). A reciprocal role between TNF-α and IL-10 has also been suggested. Thus, the addition of anti-IL-10 antibodies to cultured mononuclear cells from RA synovium significantly increased TNF-α production, while conversely the addition of recombinant IL-10 to RA mononuclear cells from joints lowered TNF-α production (Katsikis et al. 1994). Inaccurate deviation in the type1/type2 cytokine pattern may contribute to disease. A predominant type 1 response is implicated in certain organ-specific autoimmune disorders such as RA, whereas a bias towards type 2 phenotypes is likely to be involved in certain systemic autoimmune diseases characterized by excessive AuAb production such as SLE, in which IL-10 has a critical role in the induction of AuAb production (Liblau et al. 1995; Llorente et al. 1995; Romagnani et al. 1997; Tokano et al. 1999). Cytokines appear to play a critical role, whereby their sustained production, possibly in response to a persistent antigenic challenge, could lead to chronic inflammatory changes (Brennan et al. 1992; Paludan et al. 2001). The cytokines that have been targets of particular interest and which seem to have a central role in RA and SLE are TNF-α and
IFN-γ (type 1) and IL-10 (type 2), respectively. The properties of these cytokines will be described in more detail below.

**Type 1 cytokines (TNF-α and IFN-γ)**

**TNF-α.** TNF-α, initially described by its anti-tumor activity, is a mediator of inflammation and cellular immune responses (Vassalli 1992; Vassalli et al. 1992). TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells and natural killer (NK) cells following stimulation e.g. by lipopolysaccharides (LPS). Activated macrophages are the major source of TNF-α (Vassalli et al. 1992). Two receptors for TNF-α have been described, 55 KDa and 75 KDa. The mechanisms through which TNF-α exerts its effects are manifold. TNF-α is required for a normal immune response and *in vivo* TNF-α has both beneficial and deleterious effects. However, its over-expression may mediate severe effects, as is apparent in RA patients (Feldmann et al. 1992).

**IFN-γ.** IFN-γ, which is a member of the interferons, was initially defined as a proinflammatory mediator. T-cells and NK cells are the main sources of IFN-γ production. As a consequence of phagocytosis macrophages secrete IL-12, which is necessary for the induction of IFN-γ (Figure 2). IFN-γ has antiviral and antiparasitic activities and appears to have immunomodulatory functions. It also inhibits the proliferation of a number of normal and transformed cells. IFN-γ acts synergistically with TNF-α and TNF-β (also known as lymphotoxin-alpha, LT-α) in inhibiting the proliferation of various cell types (Balkwill 1989; Balkwill 1989; Farrar et al. 1993). IFN-γ acts synergistically with IL-2 that induces its production, and at the same time INF-γ appears to be required for the expression of IL-2 receptors on the cell surface of T-cells. IFN-γ may also regulate the expression of MHC class I and class II genes (Snapper et al. 1997).

**Type 2 cytokines (IL-10)**

IL-10 is a molecule that has profound anti-inflammatory and immunoregulatory effects. In addition to the anti-inflammatory functions such as inhibition of TNF-α production and induction of endogenous TNF-α inhibitors (Di Santo et al. 1995), IL-10...
has B cell stimulatory effects. In humans, IL-10 is produced by T-cells as well as by LPS-activated monocytes, macrophages and mast cells (Llorente et al. 1995).

IL-10 plays a crucial role in the regulation of immune responses via various mechanisms. IL-10 sustains viability of B-cells in vitro and stimulates and promotes their differentiation (Rousset et al. 1992). In activated B cells IL-10 induces the secretion of IgG, IgA and IgM. This effect is synergised by IL-4 and antagonised by TGF-β (Defrance et al. 1992; Roussel et al. 1992). It has also been shown that IL-10 enhances proliferation and Ig production by B cells by preventing apoptosis of germinal center B cells (Roussel et al. 1992; Itoh et al. 1995; Llorente et al. 1995; Pound et al. 1997). An in vitro study of IL-10 revealed that IL-10 had enhancing effects on anti-dsDNA antibody production (Llorente et al. 1995). In experimental models (Lupus-prone NZB/WF1 mice) treatment with anti-IL-10 has been reported to delay autoimmunity, decrease anti-dsDNA and subsequently to decrease the severity of renal disease (Ishida et al. 1994). IL-10 inhibits the synthesis of a number of cytokines such as INF-γ, IL-2 and LT-α in type 1 T-cell subpopulations (Fiorentino et al. 1991).

**TGF-β1**

TGF-β family proteins are pleiotropic, secreted signaling molecules with potent immunoregulatory and anti-inflammatory properties. TGF-β is produced by cells of the leukocyte lineage including, lymphocytes, macrophages and dendritic cells. TGF-β promotes fibrosis by its stimulatory effects on synthesis of extracellular matrix (Wahl 1994), and also in pulmonary fibrosis in other inflammatory condition (Wahl 1994). TGF-β is present in synovial membranes in RA (Ulf gren et al. 1995).

**Cytokines in RA, SLE, MCTD and PM/DM**

Cytokines are involved in general inflammation, in triggering and/or maintenance of multi-system dysfunctions in chronic inflammatory disorders. The importance of cytokines as possible mediators of tissue injury in connective tissue disorders is exemplified by the ability of TNF-α to stimulate resorption and inhibition of proteoglycan synthesis in cartilage (Hickery et al. 1990). IFN-γ participates in inflammatory responses through its ability to enhance TNF-α production and activity.
Another pathogenic role of cytokines in rheumatic disorders is manifested by IL-10, which has immunosuppressive effects but it also has a stimulatory effect on B cells through driving AuAb production (Llorente et al. 1995; Cross et al. 1999). Increased production and activation of latent TGF-β has been linked to immune defects associated with autoimmune disorders and to fibrotic complications associated with chronic inflammatory conditions. An increased production of cytokines has been observed locally in the joints in RA, and is accompanied by the occurrence of inflammatory lymphocytes and monocytes (Ulfgren et al. 1995).

**TNF-α.** Increased levels of TNF-α have been detected in peripheral blood, synovial fluid and synovial tissue in patients with RA (Buchan et al. 1988; Saxne et al. 1988; Firestein et al. 1990). TNF-α shares many proinflammatory actions with IL-1. TNF-α was also determined to be an immunomodulating molecule in patients with RA since hypo-responsiveness of synovial T cells has been reported to be induced by chronic TNF-α signaling (Lai et al. 1995). The same process of suppression has been confirmed by single infusion of anti-TNF-α antibodies, which was found to be sufficient to restore peripheral T cell responses to recall antigens and/or mitogens (Lorenz et al. 1996; Berg 2000). The TNF-α molecule has also become one of the first target molecules for new anti-inflammatory therapies in RA and Crohn’s disease (Feldmann et al. 1997; Maini et al. 1997; Feldman et al. 1998; Sandborn et al. 1999).

The role of TNF-α in SLE has been more controversial. In a trial of TNF blockade with RA patients, development of anti-dsDNA antibodies was reported as well as occasional patients with lupus symptoms (Maini et al. 1994). On the contrary, more recently increased serum levels of TNF-α have been reported in subsets of SLE patients with cardiovascular disease suggesting a role in disease mechanisms at least in some patients with SLE.

Whether TNF-α has a role in the underlying disease mechanisms in myositis or MCTD is less well understood. TNF-α was observed in muscle tissue of patients with IIM in several studies (Lundberg et al. 1995; Tews et al. 1996; De Bleecker et al. 1999). There are no reports of TNF-α in MCTD.

**IFN-γ.** Several reports of the abnormalities in the type 1 cytokine IFN-γ in different autoimmune diseases have been published. These abnormalities include
increased serum levels of IFN-γ (Funouchi et al. 1991). High circulating levels of IFN-γ have been reported in SLE (Kim et al. 1987) and presence of IFN-γ was observed in the synovial tissue of RA patients (Ulfgren et al. 1995). Treatment with recombinant IFN-γ in RA has been the subject of several clinical trials that revealed both beneficial (Cannon et al. 1990; Pincus et al. 1990) and deleterious effects as it induced SLE in some RA patients (Graninger et al. 1991).

**IL-10.** Increased production of IL-10, which has a stimulating effect on B-cells and AuAb production, has been observed in patients with several autoimmune diseases such as SLE and SS (Llorente et al. 1994). In SLE the increased IL-10 production seems to be unrelated to disease activity (Grondal et al. 2000). Moreover, increased IL-10 production has also been detected in both sanguineous and non-sanguineous relatives of SLE patient suggesting that the increased IL-10 production in SLE could either be caused by genetic or environmental factors (Jones et al. 1992; Llorente et al. 1997; Grondal et al. 1999).

In RA, increased IL-10 levels were described in serum and synovial fluid from inflamed joints (Cush et al. 1995). It seems that IL-10 functions as an anti-inflammatory molecule in the inflamed joint, since the addition of anti-IL-10 mAb to synovial cell cultures increased the production of pro-inflammatory cytokines such as TNF-α (Isomaki et al. 1996). IL-10 has also been shown to ameliorate arthritis in animal models (Walmsley 1996).

As both MCTD and PM/DM diseases are characterized by AuAb production it is likely that IL-10 could also have a role in these diseases. At present there are no reports of serum levels or the role of IL-10 in MCTD and PM/DM patients. Only occasional patients have been included in studies with SLE patients and RA patients. Thus no conclusions about the role of IL-10 in MCTD or myositis can be drawn from these studies.

**TGF-β1.** TGF-β cytokine has anti-inflammatory and profibrotic effects and is likely to be involved in the disease mechanisms of at least some chronic inflammatory diseases. Increased expression of TGF-β in rheumatoid synovium in RA patients has been reported (Cheon et al. 2002; Yamanishi et al. 2002). In SLE a role of TGF-β in the
development of lupus nephritis has been suggested (Yamamoto et al. 2000). The immunomodulatory role of TGF-β1 in idiopathic inflammatory myopathies (IIM) remains to be elucidated. Increased expression of TGF-β in muscle biopsies of patients with IIM has been reported (Lundberg et al. 1995; Lundberg et al. 1997). A similar pattern of expression of mRNA of TGF-β1 in muscle tissues in both PM and DM was observed (Lundberg et al. 1995), while others have reported that TGF-β1 mRNA was significantly higher in DM, but not PM, compared to controls (Confalonieri et al. 1997).

To study the role of cytokines in disease mechanisms of chronic rheumatic diseases, and in particular of the AuAb production, I have investigated patients with different rheumatic diseases, which are all characterized by AuAb production, but with different profiles. Among the various autoimmune disorders four related autoimmune rheumatic disorders will be discussed in my thesis, MCTD, SLE, RA and idiopathic inflammatory sympathies (IIM) (myositis). These disorders share some clinical and immunological features but each of them is also characterized by some distinct phenotypes.

**C-reactive protein**

C-reactive protein (CRP) is one of the acute phase proteins (APP) that are mainly synthesized by the liver. Changes in APP occur in response to infections or tissue injury (Koj 1970). It has also been shown that changes in APP occur in chronic rheumatic diseases. It has been reported that CRP increases the mRNA expression of IL-1 and TNF-α in human macrophages. Cytokines are the main regulators of APP. Potent inflammatory cytokines in the upregulation of CRP are IL-6, IL-1 and TNF-α. A remarkable fall in circulating levels of CRP following anti-TNF-α therapy in RA has been reported (Charles et al. 1999).

**Autoimmune disorders**

*Mixed connective tissue disease*

Mixed connective tissue disease (MCTD), which was initially described by Sharp et al in 1972, is interesting to study in the perspective of AuAbs and cytokines as this
disease entity is clinically characterized by combined features of other defined autoimmune disorders such as SLE, systemic sclerosis (SSc) and PM (Sharp et al. 1972). The clinical features that are characteristic of MCTD are puffy hands, Raynaud’s phenomenon, arthritis, sicca symptoms and myositis. The characteristic immunological markers are presence of AuAbs such as anti-U1 RNP, but also high serum levels of immunoglobulins and rheumatoid factor (RF). In contrast, anti-Sm antibodies are not found in this disorder (Bennett et al. 1980; Lundberg et al. 1991; Hassan et al. 1998). A genetic association with HLA-DR4 has been suggested but the studies of genetics in MCTD are limited (Ruuksa et al. 1992; Hameenkorpi et al. 1993). The Alarcon-Segovia classification criteria are the most commonly used diagnostic criteria for MCTD (Table 2) (Alarcon-Segovia 1987).

Table 2. The Alarcon-Segovia classification criteria for the MCTD

<table>
<thead>
<tr>
<th>Serologic criterion:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-RNP at a hemagglutination titer of 1:1600 or higher</td>
</tr>
<tr>
<td>Clinical criteria:</td>
</tr>
<tr>
<td>a. Edema of hands</td>
</tr>
<tr>
<td>b. Synovitis</td>
</tr>
<tr>
<td>c. Myositis</td>
</tr>
<tr>
<td>d. Raynaud’s phenomenon</td>
</tr>
<tr>
<td>e. Acrosclerosis</td>
</tr>
</tbody>
</table>

Diagnosis of MCTD requires a serological criterion plus at least three clinical criteria (among them at least b or c). (Alarcon-Segovia 1987).

The prognosis of MCTD patients was originally reported to be benign with a good response to low doses of corticosteroids, although later it became evident that vital organs may be affected particularly the heart and the lungs. Neurological abnormalities were also more affected, particularly the heart and lungs. Neurological abnormalities were also more frequent than previously thought (Bennett et al. 1978), while the kidneys are rarely involved. The arthritis was originally reported as non-erosive, but later an erosive and sometimes deforming RA-like polyarthritis has been observed in patients with MCTD (Ramos-Niembro et al. 1979; Bennett et al. 1980; Piirainen et al. 1990; Lundberg et al. 1991), and this has been reported to associate with presence of RF as well as U1 RNP antibodies (Piirainen 1990; Piirainen et al. 1990).
Poly- and dermatomyositis

Polyomyositis (PM) and dermatomyositis (DM), which are chronic systemic inflammatory disorders, are the most common forms of IIM. The hallmarks of these diseases are symmetric proximal limb and neck weakness, sometimes associated with muscle pain, elevated serum levels of muscle enzymes and immune dysregulation including, AuAb production and infiltration of lymphocytes and macrophages into muscle tissue (Arahata et al. 1984; Engel et al. 1984; Dalakas 1991; Dalakas 2001). Diagnosis of PM/DM is based on criteria suggested by Bohan and Peter (Table 3) (Bohan et al. 1975; Bohan et al. 1975).

Two types of AuAbs were identified in the sera of IIM patients, myositis-specific AuAbs (MSAs) and myositis-associated autoAbs (MAAs) (Miller 1993; Targoff 2000). MAAs (such as ANA, anti-PM/Scl, -U1snRNP, -Ro60/SSA, -Ro52/SSA and –

Table 3. Proposed diagnostic criteria for polymyositis (PM) and dermatomyositis (DM)

| 1. Symmetric proximal muscle weakness. |
| 2. Elevated muscle enzymes.          |
| 3. Myopathic EMG abnormalities.      |
| 4. Typical changes on muscle biopsy. |
| 5. Typical rash of dermatomyositis.  |

- PM diagnosed as definite with 4 criteria out of criteria 1-4 (4/1-4) or probable with 3/1-4 or possible with 2/1-4.
- DM diagnosed as definite with rash + 3/1-4 criteria or probable with rash + 2/1-4 criteria or possible with rash + 1/1-4 criteria.

Bohan A, Peter JB. 1975

La/SSB antibodies) are not specific for IIM and are often encountered in other rheumatic disorders without signs of myositis. The MSAs consist of three groups of AuAbs, one directed to a group of synthetase enzymes (tRNAs), signal recognition particles (SRP) and nuclear helicase/ATPase Mi-2 (Targoff 1994; Plotz et al. 1995).
An increased expression of the cytokines IL-1α, IL-1β and TNF-α has been recently demonstrated in muscle tissue of patients with PM/DM suggesting that these proinflammatory cytokines may have a role in the disease mechanisms of myositis (Lundberg et al. 1995). Neither the etiology nor the susceptibility factors for these diseases are known.

**Systemic lupus erythematosus**

Systemic lupus erythematosus (SLE) is another chronic systemic autoimmune disease of unknown etiology. SLE is a disorder with a great heterogeneity in clinical expression and serological factors (Smolen et al. 1985; Swaak et al. 1999). Every organ can be involved and the most frequently affected organs are the skin, joints, kidneys, heart and CNS. Clinical features characteristic of other CTDs occur occasionally in SLE, such as erosive arthritis (Isenberg et al. 1994) or myositis (Tsokos et al. 1981). Interestingly, SLE patients with muscle involvement commonly have a benign form of the disease with Raynaud’s phenomenon, antibodies to U1-snRNP and a lower risk of renal manifestations (Fries et al. 1975; Smolen et al. 1985). The ARA (American Rheumatism Association) criteria are used for diagnosis (Tan et al. 1982). Several disease activity indices, such as British Isles Lupus Assessment Group (BILAG), SLE Disease Activity Index (SLEDAI) and Systemic Lupus Activity Measure (SLAM), have been developed (Liang et al. 1988).

One of the characteristic features of SLE is B cell hyperactivity, often with a pronounced production of AuAbs, but which mechanisms promote B cell activation are not known. However, cytokines such as IL-10 are believed to be involved (Llorente et al. 1995; Cross et al. 1999). The characteristic AuAbs of SLE are antinuclear antibodies, particularly antibodies against anti-dsDNA, but also anti-ssDNA and anti-histone antibodies. AuAbs to other cellular and cytoplasmic components could also be detected, such as anti-Sm, which is pathognomonic for SLE, but rarely detected. Anti-U1RNP, anti-Ro/SSA, anti-La/SSB antibodies and RF can also be detected in SLE (Tan 1989; von Muhlen et al. 1995).
Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune rheumatic disease mainly affecting peripheral joints. Persistent joint inflammation leads to articular pain, swelling, cartilage erosion and eventual joint deformity and disability. In some RA patients, organ systems in addition to the joints become inflamed. RA is characterized serologically by presence of high titers of IgM antibodies against IgG (rheumatoid factor, RF), although RF of other isotypes can be present. The diagnosis is based upon classification criteria, the ARA (American Rheumatism Association) criteria for RA, which include both clinical and serological findings (Arnett et al. 1988).

An association between connective tissue diseases such as myositis, MCTD, RA, SLE, SSc and Sjögren’s syndrome (SS) is frequently encountered (Sharp et al. 1972). This indicates that there could be common immunopathogenic mechanisms in all these related autoimmune rheumatic disorders resulting in different disease outcomes, which may be determined in genetically different and susceptible individuals with different environmental risk factors.

The mechanisms behind the increased cytokine production in inflammatory diseases are unknown and both genetic and/or environmental factors could be involved. The polymorphisms in the regulatory regions of cytokine genes have been the subject of intense interest as potential markers of disease susceptibility. Little is known about the molecular mechanisms through which they may influence cytokine gene expression, although family studies show that up to 60% of the variability in TNF production between individuals may be genetically determined (Westendorp et al. 1997).

Genetic considerations

Hypothetically, the genes for susceptibility to different diseases are sometimes shared but sometimes different although the phenotype (clinical symptoms and AuAbs) can be similar, as indicated by family studies in man, animal studies and genome-wide scans (Becker et al. 1998). For example, in families with sporadic IIM cases a high frequency of other autoimmune diseases was evident (Genin et al. 1998). Increased knowledge of the role of these genes in different diseases is likely to improve our understanding of disease mechanism in autoimmune diseases.
General genetic aspects

Genetic polymorphisms are defined as variations in DNA sequences that are observed in 1% or more of the population. Genetic polymorphisms may alter protein structure and function through single nucleotide base substitutions in a gene’s coding region and may increase or decrease gene expression either by affecting mRNA stability when occurring in a gene’s 3’ untranslated region or by altering transcription factor binding when occurring in the 5’-promoter region (Weber et al. 2001). Genetic polymorphisms are most often assessed using association studies (Risch et al. 1996).

Single nucleotide polymorphisms (SNP) are stable, inherited, biallelic, single base pair differences which are present in the human genome at the density of 1 to 10 per 1000 nucleotides (The International SNP 2001). SNPs are potentially powerful tools with which to analyze genetic variability.

Linkage disequilibrium (LD) means that particular combinations of alleles of various loci (i.e. closely located in the chromosome), for instance HLA-DR4 and HLA-DQ8, remain linked together in a haplotype with very little recombination in between.

Ancestral Haplotype. In light of linkage disequilibrium the closely located genes in the MHC region on chromosome 6 are carried by the highly conserved ancestral haplotype 8.1 (HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2) reviewed in (Price et al. 1999). The ancestral haplotype (AH) 8.1 is common in Caucasian populations and was previously demonstrated to confer susceptibility to autoimmune diseases such as IDDM (Degli-Esposti et al. 1992), SLE (Wilson et al. 1994) and SS (Ricchiuti et al. 1994).

An epistatic phenomenon is classically defined as a genetic interaction in which the genotype at one locus affects the phenotypic expression of the genotype at another locus. The epistasis between two susceptibility alleles leads to a greater effect in disease severity than would be predicted by simply adding together their individual phenotypes.
**Immunogenetics**

**MHC genes**

The major histocompatibility complex (MHC) in humans named human leukocyte antigen (HLA) complex encompasses approximately 4 Mb (4 million base pairs) on the short arm of chromosome 6.

**Figure 3. Major histocompatibility complex (MHC) region**

MHC region is divided into three non-overlapping regions (Figure 3) called MHC class I (1Mb), class II (1Mb) and class III (2Mb) (The MHC Sequencing 1999). There are approximately 180 genes in MHC class I (Shiina et al. 1999), around 40 genes in class II (Beck et al. 1999) and 70 genes in class III (Aguado et al. 1996).

**MHC class II**

The three main loci of HLA class II, namely HLA-DR, -DQ and –DP, are highly polymorphic, while others (HLA-DM and –DO) are not. HLA-DR molecules are heterodimeric proteins. The α chain is encoded by the DRA gene and shows very limited polymorphism. Only two alleles of the DRA gene has been found; DRA*0101 and DRA*0102. The HLA-DR β chain is encoded by the DRB locus and 9 DRB loci (DRB1-9) have been defined, of which the DRB1 locus shows the highest polymorphism with more than 200 known allelic variants. Alleles of DRB1 associate with susceptibility to many autoimmune diseases.
Association of MHC class II genes with autoimmune diseases

Among Caucasian patients with SLE, association of HLA-DR3 alone (Fielder et al. 1983; Reveille et al. 1991) or DR3 and DR2 (Black et al. 1982; Howard et al. 1986; So et al. 1990) has been reported. Recently, a single-nucleotide polymorphism (SNP) in intron 3 of exon 4 of Ro52 gene on chromosome 11 (Frank et al. 1993) was found to be strongly associated with the presence of anti-Ro52 kDa AuAbs in primary SS (Nakken et al. 2001).

Genetic susceptibility in RA has been associated with certain DRB1 alleles: DRB1*0401, 0404 and 0101, while DRB1*0402 allele is not associated with RA and might even protect against this disease (Nepom 1998).

To study the role of genetics in IIM both multicase family studies and candidate genes studies (MHC and non-MHC genes) have been performed. Early studies of MHC genes in IIM suggested that HLA-DRB1*0301 is a risk factor for both sporadic and familial cases (Hirsch et al. 1981; Shamim et al. 2000). Serological typing methods were used in most of earlier studies.

An association between MCTD and HLA has been investigated by serological typing and an increased frequency of HLA-DR4 was observed in Caucasian MCTD patients (Genth et al. 1987; Black et al. 1988; Kaneoka et al. 1992) while DQ3 was associated with MCTD patients in a Japanese study (Nishikai et al. 1985). In another Japanese study where HLA-typing was performed by analysis of DNA, MCTD was associated with the HLA-DRB1*0401-DRB4*0101-DQA1*03-DQB1*0301 haplotype (Dong et al. 1993). In Mexican MCTD patients an increased frequency of DQB1*0501 and DR1, but not DR4 was observed (Weckmann et al. 1999).

In many autoimmune disorders different HLA class II alleles could serve as risk factors for the development of different AuAbs. Thus, in SLE many genetic studies have shown that the MHC genes strongly influence anti-Ro/SSA production (Wilson et al. 1994). In IIM antisynthetase AuAbs were associated with DRB1*0301 and DQA1*0501 in USA Caucasian patients, while in African Americans the presence of these antibodies associated with increased frequency of DQA1*0501 and/or DQA1*0401, but DRB1*0301 was not identified as a risk factor (Arnett et al. 1996). In MCTD, HLA-DR4 primarily noted to be related to U1-snRNP antibody formation and not to disease
development (Genth et al. 1987). In another study of MCTD the presence of AuAbs against the 70 kDa polypeptides of the U1 RNP (U1-70- kDa) was associated with DR4 and DR2 in MCTD patients (Kaneoka et al. 1992). The functional role of these associations between HLA and AuAbs is unclear, however.

The importance of HLA-DR molecules for disease susceptibility is indicated by their ability to present peptides to T cells. The DR molecules might play pathogenic roles through presentation of self-antigens (Chaplin et al. 1988). The HLA molecule may thus be directly involved in the pathogenesis of autoimmune diseases such as RA, SLE, myositis and MCTD. RA-associated HLA-DR molecules share specific sequence polymorphisms i.e. the RA-associated DRB1 alleles (DRB1*0101, 0401 and 0404) encode DR β chain sequence that shares the stretch of amino acids between positions 67-74 (i.e. 24 nucleotides). This shared sequence differs from the DR β chain, encoded for example by the non-RA associated DRB1*0402 allele. The shared epitope hypothesis originally stated that a shared motif in protein structure is likely to be of importance for the function of the MHC class II molecules and thus, the inheritance of susceptibility to RA (Gregersen et al. 1987; Seyfried et al. 1987). The shared epitope plays a role in antigen presentation. It contributes to the formation of the peptide-binding groove of the RA-associated HLA-DR, which of critical importance for the T-cell recognition of the HLA-peptide complex. Whether the shared epitope is important in other autoimmune diseases is not known.

Based on linkage disequilibrium between HLA-DR and certain HLA-DQ alleles (such as DQ7 and DQ8) it has been suggested that MHC class II-associated susceptibility to RA is not conferred by HLA-DR alleles separately, but rather by HLA-DQ alleles (Zanelli et al. 1995; Zanelli et al. 1998). The importance of HLA-DR-associated susceptibility to autoimmune diseases could also be due to its association through linkage disequilibrium with other functional genes such as the TNFA gene.

**MHC class III**

The MHC class III region, spanning approximately 760 Kb, is characterized by a remarkably high gene density with 59 functional genes. It has been suggested that
susceptibility loci to many autoimmune rheumatic diseases such as TNFA and TNFA-linked microsatellites loci exist in this region.

**TNFa microsatellite.** Five microsatellite repeats near the TNFA gene (TNFa, to TNFe) have been reported. Thirteen alleles of the dinucleotide repeat were identified for TNFA microsatellite (Nedospasov et al. 1991). In vitro studies have shown that TNF-α production by peripheral blood monocytes varied with different TNF microsatellite alleles (Pociot et al. 1993). The polymorphic TNF microsatellite markers associated with high levels of TNF-α production are TNFa2, TNFc2 and TNFd3 alleles (Pociot et al. 1993; Turner et al. 1995). Microsatellite repeats near the TNFA gene (Figure 3) have also been shown to be associated with susceptibility to autoimmune diseases. Thus, the TNFa6 allele was associated with susceptibility and severity to RA (Hajeer et al. 1996; Mulcahy et al. 1996). Moreover, TNFa6 in the shared-epitope positive female RA patients was associated with high disease activity (Mattey et al. 1999). A recent study demonstrated that not TNFA promoter polymorphisms, but TNFa6/TNFb5 haplotypes were significantly associated with susceptibility to RA, even if these haplotypes lacked the HLA-DRB1*04 alleles, suggesting that the TNFa/TNFb haplotype is an independent marker of RA susceptibility (Martinez et al. 2000). In SLE, association with both –308TNFA and TNFa microsatellite has been reported (van der Linden et al. 2001). No analyzes of TNFa microsatellites have been reported in patients with myositis or MCTD.

**TNFA.** TNFA gene is mapped on chromosome 6 within the MHC class III region (Figure 3). Several single nucleotide polymorphisms (SNPs) in the human TNF locus have been reported (Fugger et al. 1989; Fugger et al. 1989; Fugger et al. 1989; Webb et al. 1990). A G → A substitution in –308 position of the TNFA gene results into two alleles (-308ATNFA or TNF2 allele and –308GTNFA or TNF1 allele). It was reported that TNF2 associated with increased TNF-α production (Waldron-Lynch et al. 2001). It has been suggested that on the HLAB8-DR3 haplotype, the TNF2 may play a role in SLE susceptibility, but was not found to be associated with AuAb production such as anti-Ro and anti-La antibodies (Wilson et al. 1994). TNF2 was not associated with disease susceptibility in patients with SSc (Pandey et al. 1999) or with RA (Brinkman et al. 1996;
Field et al. 1997) nor to clinical and radiological outcome in patients with RA (Lacki et al. 2000). The effect of the allele frequency of TNFA gene on inter-individual variations of cytokine production in patients with MCTD and adult PM/DM has not been clarified. Such knowledge may provide a clue as to why patients produce inappropriate quantities of cytokines and whether genetic influence is involved in cytokine production.

MHC class I-related chain gene (MICA)

A distinct family of MHC class I genes has recently been identified within the human MHC class I region, named MHC class I chain-related (MIC) genes (Bahram et al. 1994) and is associated with autoimmune diseases (Table 4). There are two functional genes (MICA and MICB) and three pseudo-genes (MICC, MICT and MICE). The MICA gene is located between the TNFA and the HLA-B genes. MICA molecule has a unique pattern of tissue expression and appears to be highly flexible and polymorphic, although the functional relevance and implications of this polymorphism have yet to be fully discerned (Das et al. 2001; Stephens 2001).

No clear functional role for the MICA molecule has been determined, although several functions have been proposed (Bahram et al. 1994). This gene is distinguished by its preferential expression in keratinocytes and monocytes, but not in T cells or B cells (Zwirner et al. 1999).

It has recently been reported that MICA also behaves as a stress induced molecule, MICA molecule, being reported to be induced on the surface of dendritic and epithelial cells by infection with M. tuberculosis in vitro and in vivo (Das et al. 2001). Association studies of the polymorphism (trinucleotide repeat) in exon 5 of MICA gene encoding a transmembrane protein of MICA molecule showed that this gene is a strongly associated with certain autoimmune diseases (listed in Table 4). This gene could also be of interest in MCTD or myositis, but there have been no data published on the MICA gene in these two patient populations.
Table 4. MICA alleles are associated with some autoimmune diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Allele association</th>
<th>Reference</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDDM</td>
<td>MICA5 positively associated and</td>
<td>(Gambelunghe et al. 2000)</td>
<td>Italian</td>
</tr>
<tr>
<td></td>
<td>MICA6 negatively associated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behcet disease</td>
<td>MICA6 positively associated</td>
<td>(Mizuki et al. 1997)</td>
<td>Japanese</td>
</tr>
<tr>
<td>AS</td>
<td>MICA4 positively associated</td>
<td>(Goto et al. 1997)</td>
<td>Caucasian</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>MICA5.1 positively associated</td>
<td>(Gambelunghe et al. 1999)</td>
<td>Italian</td>
</tr>
<tr>
<td>RA</td>
<td>MICA6 negatively associated (in SE⁺ RA group)</td>
<td>(Martinez et al. 2001)</td>
<td>Spain</td>
</tr>
<tr>
<td>JIA</td>
<td>MICA4 positively associated</td>
<td>(Nikitina Zake et al. 2002)</td>
<td>Latvian</td>
</tr>
</tbody>
</table>

IDDM = Insulin-dependent diabetes mellitus, JIA = Juvenile idiopathic arthritis, AS = Ankylosing spondylitis, RA = Rheumatoid arthritis, SE⁺ = Shared epitope positive.

**Non-MHC genes (IL10 and TGFB1)**

Associations with non-MHC genes have also been confirmed in some autoimmune diseases. An association between SLE and IL10 gene polymorphism has been reported (Turner et al. 1997; Hajeer et al. 1998; Cantagrel et al. 1999). Studies of non-MHC loci are limited in IIM and were mainly reported from juvenile dermatomyositis (JDM). In addition to the more studied MHC cytokine gene, TNFA, the non-MHC gene frequency was analyzed for IL1 (Pachman et al. 2000; Shamim et al. 2000; Shamim et al. 2000). IL1RN VNTR (variable number tandem repeats) polymorphism was the first non-MHC genetic risk factor identified for JDM and different alleles may confer susceptibility for different ethnic groups (Rider et al. 2000). One explanation to the limited number of studies, which have focused on non-MHC genes such as cytokines genes could be that very large populations of patients and matched controls will be needed for efficient analysis of these genes, which have much weaker associations in comparison to MHC genes.

Both IL10 and TGF-β1 genes may be of interest in MCTD and myositis due to their clinical phenotypes with overlap syndrome and fibrosis, particularly in the lung.
IL10 gene

The gene for IL-10 (IL10) has been mapped on chromosome 1 (Esksdale et al. 1997; Esksdale et al. 1997). The promoter region of the IL-10 gene contains several SNPs e.g. G/A at -1087, C/T at -824 and C/A at -597. These three SNPs form 3 haplotypes, GCC, ACC and ATA. The three haplotypes are known to differ in their effect on the transcription of the IL10 gene. The G/A polymorphism at position −1087 has been linked to high/low IL10 producer status, respectively. Functional significance of the −1087 polymorphism has been confirmed. Transient transfection studies in a B cell line indicated that the −1087IL10A allele confers a two fold increase in transcriptional activity of the IL10 gene promoter compared to the G allele. There was marked inter-individual variations in IL10 production by peripheral blood mononuclear cells in vitro, with no consistent effect of genotype (Rees et al. 2002).

Several studies have reported associations between IL10 polymorphism and risk of a diverse range of diseases including SLE (Gibson et al. 2001) and RA (Kaluza et al. 2001). Studies of IL10 gene in patients with IIM and MCTD are lacking.

TGFB1 gene

The gene for TGF-β1 (TGFB1) has been mapped on the long arm of chromosome 19 (Fujii et al. 1986). Several TGFB1 gene polymorphisms have been described of which two signal sequence polymorphisms at +869 (at codon 10) and +915 (at codon 25) have been implicated in different diseases (Awad et al. 1998; Suthanthiran et al. 1998). The +915 polymorphism at codon 25 is a G→C substitution, resulting in an arginine→proline change, is associated with interindividual variations in levels of TGF-β1 production. There is a strong linkage disequilibrium between the polymorphisms at codon 10 (+869) and at codon 25 (+915). A recent study reported that SSc patients are genetically predisposed to high TGF-β1 production where there was a significant increase of allele C at codon 10 (+869 polymorphism), but there was no difference in allele frequency between SSc patients and controls at codon 25 (+915) (Crilly et al. 2002). In vitro experiments revealed that the homozygous state of TGFB1 gene polymorphism (GG genotype) at +915 of codon 25 resulted in more TGF-β1 secretion from stimulated
lymphocytes than heterozygotes and this allele was significantly associated with post transplant fibrotic pathology of the lung (Awad et al. 1998). Studies of gene polymorphisms of TGFβ1 gene in patients with IIM or MCTD are lacking.

An association between IL-10 and TGF-β1 at protein levels was established (Defrance et al. 1992). The opposite is true for IL-10 and TNF-α (Katsikis et al. 1994), but whether this refers to genetic basis is not known.

THE PRESENT INVESTIGATIONS

Knowledge about the common mechanisms responsible for immune dysregulation in autoimmune rheumatic diseases such as high AuAb production will result not only in an earlier and more accurate diagnosis, but may also lead to the development of novel therapeutic approaches aimed at modulating the expression of these markers. The ultimate hope is that this will provide fundamental insights into molecular pathogenesis of disease and eventually lead to better treatment and prevention.

Considering the mechanisms responsible for immune dysregulation in autoimmune rheumatic diseases, MCTD, myositis and SLE, cytokines such as TNF-α, IL-10 and TGF-β1 and AuAbs, U1snRNP, anti-Jo-1, anti-Ro/SSA and anti-La/SSB antibodies, could all play key roles in these diseases. Certain susceptibility genes may be shared among different autoimmune diseases. Polymorphisms of genes within the MHC region (MHC class I, MHC class II and MHC class III) as well as outside this region (non-MHC genes) have been a fundamental issue in these autoimmune disorders.

AIMS

The general aim of this thesis was to investigate immunological and genetic pathways in the development of autoimmune diseases, specifically MCTD and PM/DM. Particular focus was in on the potential role of cytokines in AuAb production and the cytokine gene polymorphisms in patients with clinical phenotypes and AuAb profiles that are partly shared and partly different. For this we have chosen to investigate patients with MCTD, myositis, SLE and RA with a focus on AuAbs that are characteristic for these diseases, namely anti-U1-RNP, -Ro/SSA/-La/SSB and Jo-1 AuAbs with the aim to relate these to disease activity, clinical phenotypes and serum levels of cytokines.
Specific aims in this thesis were to:

1. Investigate the relationship between patterns of AuAbs and clinical features as well as disease activity in patients with MCTD, SLE and poly- and dermatomyositis.

2. Investigate AuAb pattern, clinical features and disease activity in relation to serum levels of type 1 (TNF-α) and type 2 cytokines (IL-10), in patients with MCTD and poly- and dermatomyositis.

3. Analyze MHC gene polymorphisms in the MHC class III region (-308 TNFA gene and TNFa microsatellites), MHC class II region (HLA-DRB1, -DQA and -DQB) and MHC class I-related gene (MICA microsatellites) in relation to clinical phenotypes, AuAbs and serum levels of cytokines in patients with MCTD and PM/DM.

4. Examine non-MHC gene polymorphisms mainly at position −1087 of the IL10 gene and at +915 (codon 25) of the TGFβ1 gene and their association with the laboratory data and clinical phenotypes in patients with myositis with particular emphasis on ILD.

PATIENTS AND METHODS

A detailed description of patients and methods is provided in the individual original papers, which are referred to by their Roman numerals given on page 2. I will give a brief summary of the patients, controls and the methods used in these studies.

Patients and Controls

All the demographic data and clinical methods used to study the different patient groups included in the thesis are summarized in Table 5. The numbers of MCTD patients included in different papers are illustrated in Figure 4. The numbers of healthy controls included in all different papers are summarized in Figure 5. The same number indicates that the same group of the controls was included.
Table 5. Demographic data for MCTD, SLE, RA and PM/DM patients

<table>
<thead>
<tr>
<th></th>
<th>MCTD</th>
<th>SLE</th>
<th>RA</th>
<th>PM/DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean age/years (Paper)</strong></td>
<td>48 (I)</td>
<td>56 (II)</td>
<td>49 (I)</td>
<td>56 (I)</td>
</tr>
<tr>
<td></td>
<td>56.35 (III)</td>
<td>41.4 (IV)</td>
<td>17.4 (I)</td>
<td>17.1 (IV)</td>
</tr>
<tr>
<td><strong>Sex (F/M) (Paper)</strong></td>
<td>17/5 (I)</td>
<td>4/2 (II)</td>
<td>17/4 (I)</td>
<td>17/1 (IV)</td>
</tr>
<tr>
<td><strong>Mean disease duration /years</strong></td>
<td>11.5 (I)</td>
<td>0.3 (II)</td>
<td>16 (I)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.8-3.7 (III)</td>
<td>0.8-3.7 (IV)</td>
<td>ND</td>
<td>11 (V&amp;VI)</td>
</tr>
<tr>
<td><strong>No of patients (Paper)</strong></td>
<td>22 (I)*</td>
<td>06 (II)*</td>
<td>21 (I)</td>
<td>22 (I)</td>
</tr>
<tr>
<td></td>
<td>24 (III)*</td>
<td>18 (IV)</td>
<td>22 (I)</td>
<td>22 (I)</td>
</tr>
</tbody>
</table>

ND = Not done, No = number, * See figure 4.

**Figure 4. Total number of MCTD patients included in the thesis.**
Figure 5. Numbers of controls included in the thesis.

Methods

Immunological studies (Papers I, II, IV, V, VI, Figure 6)

All immunological markers that were studied in this thesis, as well as methods used and patient groups included are summerized in Figure 6.

Figure 6. Immunological studies in different papers
Anti-U1-RNP antibody (Papers I, II, VI)

Antibodies against antigen components within the snRNP complex were analyzed with VarELISA (enzyme-linked immunosorbent assay). The solid phase consisted of the recombinant RNP components U1-snRNP-A (33 KDa), U1-snRNP 68 KDa, U1-snRNP-C (22 KDa), both in mixture and individually, recombinant SmB’B, affinity-fractionated SmD and the purified U1-snRNP complex (RNP-Sm) from human HeLa cells. All assays were performed at Pharmacia & Upjohn, Elias Division, Freiburg.

Preparation of recombinant Ro and La proteins (Paper IV)

To perform the ELISA for analysis of anti-Ro and anti-La AuAbs we prepared recombinant proteins, Ro52, Ro60 and La proteins, as well as MaBP (maltose binding protein, wild type) as described in the original paper IV.

Anti-Jo-1 (Paper VI)

An ELISA method was used for determination of anti-Jo-1 AuAbs at the Department of Clinical Immunology, Karolinska Hospital, Stockholm.

Immunoglobulins and CRP (Paper I)

Total immunoglobulin levels of IgG, IgA and IgM classes as well as CRP were analyzed by nephelometry. The analyses were performed at the department of Clinical Chemistry at the Karolinska Hospital, Stockholm.

Serum levels of cytokines (ELISA, in all papers)

An in-house ELISA constructed as described in paper I was used for the measurement of serum levels of IL-10 and IFN-γ. For determination of TNF-α and IL-10 in the other papers and TNF-α in paper I, high sensitivity, commercial ELISA kits were used (Biosourcer, Camarilla, CA, USA).
Clinical studies (Papers I, II, IV, VI, Figure 7)

Figure 7 represents some of the important clinical data that I have studied in this thesis.

Figure 7. Clinical data used in the thesis.

Genetic studies (Papers III, V and VI, Figures 8 and 9)

The genetic markers both in MHC and non-MHC regions that I have studied in this thesis are summarized in figure 8.

Figure 8. Genetic studies of all markers analyzed in the thesis.
DNA extraction

DNA was extracted from whole blood using either standard phenol-chloroform method or the “salting-out” method (Padyukov et al. 2001).

Genotyping of HLA (HLA-DRB1, -DQA1, -DQB1)

For genotyping of HLA-DQ we used SSOP-PCR (sequence specific oligonucleotide probes for PCR), while for HLA-DRB1 genotyping SSP-PCR (sequence specific primers for PCR) DR low-resolution typing kits were used for analysis (Olerup SSP™ DR low-resolution kits).

Genotyping of Microsatellites (TNFa and MICA gene)

DNA was amplified using SSP for MICA exon 5 and TNFa microsatellites. Reverse primers were unilaterally labeled at 5’-end with the fluorescent reagent - TET, HEX or 6-FAM. Amplified microsatellite products were identified using a Perkin-Elmer ABI prism 373 DNA sequencer and output files were analyzed with GeneScan and Genotyper software.

Genotyping of cytokine polymorphisms (papers III, V and VI, figure 9)

For determination of IL10 allele polymorphism in the promoter region at position -1087 of IL10 gene the upper primer (Padyukov et al. 2001) and lower primer (Turner et al. 1997) were used as described previously. After cutting of the amplified product (590 bp) by EcoNI enzyme homozygous GG state was identified by two fragments 280 and 310 bp, while heterozygous AG state had four fragments 310, 280, 252 and 28 bp fragments and homozygote AA had 310, 252 and 28 bp fragments.

The codon 25 polymorphism of +915TGFB1 gene was recognized by Bgl II restriction enzyme. The upper and lower primers were used as described previously (Padyukov et al. 2001). The digestion of the amplified fragment (317 bp) generated the following bands; for homozygotic GG state the 243, 60 and 14 bp bands for heterozygotic CG state the 303, 243, 60 and 14 bp bands, and for possible homozygotic state of CC the 303 and 14 bp bands.
Figure 9. The size of the fragments (bp) generated by restriction endonucleases specific for each SNP in -1087 IL10, -308 TNFA and +915TGFB1 genes.

TNFA $$\rightarrow$$ NcoI $$\rightarrow$$ 5'...C↓CATGG...3'
3'...GGTAC↑C...5'

IL-10 $$\rightarrow$$ EcoNI $$\rightarrow$$ 5’CCTNN↓AGG3’,
3’GGANNN↑NTCC5’

TGFB1 $$\rightarrow$$ BglI $$\rightarrow$$ 5’GCCNNNN↓NGG3’
3’ CGGN↑NNNNCCG5’
The alleles for –308 TNFA gene polymorphism detection was performed with primers described previously (Galbraith et al. 1995). A length fragment of 107 bp was digested with Nco I. For TNF1/TNF1 homozygotic state it generates a band of 87 bp and a low size 20 bp band fragments. For TNF2/TNF2 homozygotic state a single band 107 bp fragment is generated and for the heterozygous state, TNF1/TNF2, three fragments of 87 bp, 20 bp and 107 bp are generated.

**Statistics**

Mann-Whitney U test, Spearman Rank correlation, pair-matched Wilcoxon test, Chi-Square and Fisher exact tests were used for the statistical analyses in the different papers. A p value <0.05 was considered significant. In the genetic studies Chi-square and Fisher exact tests were used to analyze the contingency in genotype and haplotype frequencies between the patients and the controls. P values and corrected p values (Pe) were calculated. Significance was assigned for Pe of ≤ 0.05. The relative risk or odds ratio (OR) and 95% confidence interval (CI) have also been calculated. The p values in accordance with the observed numbers of alleles or genotypes were corrected by 5 for MICA alleles and by 15 for MICA genotypes, by 13 for HLA-DR4 alleles and by 28 for HLA-DR4 genotypes, by 13 for TNFa microsatellite alleles and by 3 for the genotypes of each of TNFA, IL10 and TGFB1 genes. For haplotype reconstruction we used the EM Algorithm (Schneider et al. 2000) and the P values were corrected for the maximum number of the observed haplotypes. Correspondence to the Hardy-Weinberg equilibrium was tested.
RESULTS AND DISCUSSION

Immunological studies (Papers I, II, IV, V and VI)

Autoantibodies (Papers I, II, IV, VI)

To gain an increased understanding of the AuAbs and their importance in disease mechanisms I have investigated their relation to disease activity and to cytokine serum levels in cross-sectional as well as longitudinal studies and also to some extent to genetic markers.

U1-snRNP.

Using VarELISA technique (paper II) I analyzed longitudinal samples from patients with MCTD. MCTD patients had increased levels of AuAbs to the main antigenic targets of RNP, 68 KDa, RNP-A and RNP-C. The pattern of antibody titer kinetics was parallel for anti-RNP-A, anti-68 KDa and anti-RNP-C and the correlation between each two of them was statistically significant. Variations of the antibody levels during the disease course were observed.

The SLAM score developed for SLE was used for estimation of disease activity in MCTD. By using the quantitative method, VarELISA, for estimation of the anti-U1 RNP antibody levels, a correlation was observed between both anti-68 KDa and anti-RNP–A antibody levels with disease activity in our group of MCTD patients. Decreased AuAb levels also corresponded to decreased disease activity with time. One earlier study also demonstrated that fall or disappearance of anti-U1snRNP antibodies seemed to occur in association with prolonged remission (Pettersson et al. 1986). Other studies failed to confirm this observation (de Rooij et al. 1990; ter Borg et al. 1991). Previous studies trying to examine the correlation between anti-U1snRNP antibodies and disease activity have been hampered by difficulties in both quantification of anti-U1snRNP antibodies and proper assessment of disease activity. The advantage with the VarELISA method that we used is the possibility to detect fluctuations of serum levels more easily than previously used methods. Our findings of decreased anti-U1RNP antibody levels with time, is different compared to AuAb patterns evident in RA and SLE patients, in whom
AuAbs persist for many years (paper IV). This finding could indicate an exogenous precipitating (or persisting) agent in patients who develop MCTD.

The MCTD patients in our study were also characterized by having highly increased levels of IgG compared to SLE and RA patients (paper I). The main objective of analyzing total IgG in paper II was to test whether the fluctuations of anti-U1RNP antibodies were specific or a part of a non-specific immune response. The IgG levels fluctuated with time and co-fluctuated with anti-U1RNP antibody levels in all investigated patients. This indicates that the fluctuations are not restricted to anti-U1snRNP antibody, but are part of a polyclonal B cell activity.

The IgG nature of the anti-U1snRNP antibodies and the association of various AuAbs with typical MHC class II haplotypes previously reported are characteristic of a T cell dependent immune response. T cell reactivities to snRNP polypeptides in MCTD patients (O'Brien et al. 1990; Holyst et al. 1997) were mainly of CD4+ T cells, supporting the role of T cells in the generation of B cell immune responses. Moreover, there was a correlation between the presence of AuAbs to individual snRNP polypeptides and the T cell reactivity with that same polypeptide (Holyst et al. 1997). T cell epitopes on U1-70KDa protein were recently found to be limited to five epitopes (Greidinger et al. 2002).

**Anti-Ro/SSA and anti-La/SSB AuAbs.** To further define a possible pathogenic role of AuAbs in autoimmune rheumatic diseases we chose to investigate the correlation between anti-Ro/SSA and anti-La/SSB antibody levels and to compare them with variation in clinical disease activity in longitudinally collected serum samples from patients with SLE (paper IV) and also in all patient groups studied in this thesis (Figure 10) (unpublished data, summary).

In paper IV for the first time we performed serial investigations of Ro60, Ro52 and La AuAbs in a relatively large cohort of SLE patients, comparing AuAb levels determined by ELISA to disease activity assessed by the BILAG index. A co-variation of the three investigated AuAbs was noted in most of the investigated patients (40%), especially between anti-Ro52 and anti-La antibody levels. The changes of anti-Ro/SSA and anti-La/SSB antibody levels with time were minimal in patients with SLE, with a
constant increase during several years. A subset of patients displayed more changes, but no correlation with disease activity was observed.

The demonstrated relationship between AuAbs and disease activity in our SLE patients was consistent with a previous report (Praprotnik et al. 1999). In our study antibody levels fluctuated with the global score in some patients. Renal manifestations were associated with anti-Ro60 antibodies, musculoskeletal and CNS with anti-La antibodies, while haematological involvement with anti-Ro52 antibodies. These results indicate that the production of anti-Ro/La AuAb is a pathogenic mechanisms that influences disease expression and could determine specific clinical phenotypes in patients with SLE. The association of anti-La AuAbs with CNS symptoms is in agreement with an earlier study that reported an association between La/SSB and CNS involvement in lupus patients (Swaak et al. 1990).

**Ro and La in patients with different autoimmune rheumatic diseases**

In the light of my objective to investigate immunological parameters in patients with different rheumatic diseases such as SLE, RA, MCTD, poly- and dermatomyositis, I investigated the serum levels of anti-Ro/SSA and anti-La/SSB AuAbs in all disorders.

The results (presented in Figure 10) revealed that at least some patients within all our different patient groups had higher serum levels of Ro/SSA and La/SSB compared to controls. In this current study anti-Ro52 antibodies have been detected in approximately 48% in SLE patients, 44% in PM/DM patients, 39% in MCTD patients and 23% in RA patients. With the same manner except for SLE anti-Ro60 antibodies were found in 5% in SLE patients, 7% in PM/DM patients, 12% in MCTD patients and 9% in RA patients. Anti-La/SSB antibodies have been found in patients with SLE (24%), in PM/DM patients (53%), in MCTD patients (9%) and in RA patients (18%). These results are consistent with previous studies regarding SS, SLE and RA patients. However, more poly- and dermatomyositis patients were positive for both Ro/SSA and La/SSB antibodies than previously described (Table 1).
Figure 10. Ro/SSA and La/SSB in MCTD, PM/DM, SLE, RA and controls.

Ro = Anti-Ro autoantibodies
La = Anti-La autoantibodies
In addition, studies of anti-Ro/SSA and anti-La/SSB AuAbs in patients with MCTD were very limited. In the current study most of the Ro/SSA antibodies were directed to the Ro52 antigen within the SLE patients and very few had antibodies to Ro60 antigen, which is much less than expected from previous studies (Table 1).

Our findings confirmed previous studies in lupus and added a new knowledge that patients with MCTD may have high serum levels of anti-Ro/SSA, but lower levels of anti-La/SSB AuAbs. Moreover, one may conclude that MCTD patients had similar or comparable levels of Ro/SSA as RA, SLE and myositis patients, but might differ from these diseases in lacking anti-La antibodies. The clinical relevance of these new findings remains to be elucidated.

**Cytokines (Papers I, II, V and VI)**

To further characterize the immunological parameters, cytokine pattern and their relation to AuAb production and clinical phenotypes including disease activity were investigated (paper I). Moreover, we compared the cytokine pattern with CRP and immunoglobulin levels and compared the results in MCTD, SLE and RA patients to understand if these parameters show differences or similarities between the disorders. Measurable serum levels of type 1 cytokines, IFN-γ and TNF-α, and type 2 cytokine, IL-10, were observed in all three investigated disorders. No correlation was detected between disease activity as assessed by SLAM and IFN-γ, TNF-α or IL-10 in our MCTD or SLE patients. The MCTD patients had the highest serum levels of the three investigated cytokines, followed by RA patients. Our results indicated that MCTD patients resemble RA patients regarding the cytokine pattern as the two disorders displayed increased serum levels of the three investigated cytokines, as opposed to SLE patients who had low serum levels of the three investigated cytokines. Our results about cytokines in SLE are in agreement with some previous studies concerning IFN-γ and TNF-α, but not IL-10 (Lacki et al. 1997; Grondal et al. 2000). The discrepancies regarding cytokine levels in different studies of autoimmune diseases could be explained by the differences in methods used, type of treatment or selection of patients. Our study adds new information by the finding of increased serum levels of TNF-α, IFN-γ and IL-10 in MCTD patients, although, unrelated to disease activity in our group of patients.
In our patients cohort, CRP values were increased in MCTD patients and RA patients, but not in SLE patients. A positive correlation between CRP and TNF-α was determined in RA patients, but not in SLE or MCTD patients. No correlation between CRP and IFN-γ was evident in any of the three investigated disorders.

I further aimed to elucidate the relationship between cytokine levels, AuAbs and clinical data including disease activity in a longitudinal study of patients with MCTD (paper II). IL-10 and TNF-α were selected due to the initial observation in our cross-sectional study (paper I) in which the MCTD patients had increased serum levels of both these cytokines. As an extension to our work in the first study, the cytokine results in the second study demonstrated that during the mean observation period of 11.5 years increased serum levels of both IL-10 and TNF-α could be detected, with fluctuating levels over several years in some of the investigated patients. The IL-10 and TNF-α levels were increased, unrelated to disease activity or immunosuppressive treatment. The results further demonstrated, by observation from the longitudinal curves, that the levels of the two investigated cytokines did not co-fluctuate with each other, but rather they displayed a reciprocal pattern i.e. when IL-10 was high then TNF-α was low. The reciprocal role of TNF-α and IL-10 has already been established in both RA and SLE (Katsikis et al. 1994; Maini et al. 1994).

In this study we observed that at any time point when the cytokine levels were high the antibodies levels were high, but no statistical significant correlation between cytokine levels and AuAb levels was found, although there were co-fluctuations of both IL-10 and TNF-α and AuAb levels with time. We can not draw a firm conclusion from these observations; to confirm this observation the serum samples should have been taken more frequently than the samples in our study.

In papers V and VI we aimed to further investigate the interaction between the pro-inflammatory cytokine, TNF-α, and the anti-inflammatory cytokine, IL-10 and their relationship to clinical and laboratory features. We detected significantly increased serum levels of both TNF-α (paper V) and IL-10 (paper VI) in PM/DM patients compared to controls. As TNF-α and IL-10 induce pleiotropic effects and they reciprocally affect each other (Ishida et al. 1994; Katsikis et al. 1994; Maini et al. 1994), the TNF-α/IL-10 ratio

Significantly increased TNF-α/IL-10 ratios were observed in PM/DM patients with anti-Jo-1 antibodies. We also found increased TNF-α/IL-10 ratios in patients with anti-Ro52 and RF positive individuals. We did not find any correlation between the TNF-α/IL-10 ratio and disease activity or any clinical manifestation in PM/DM. The lack of correlation between the TNF-α/IL-10 ratio and clinical manifestations or disease activity in PM/DM could be explained by the relative heterogeneity of the patient group, with different age of disease onset and different duration of the disease, or by a true absence of correlation. Our own data suggest that not TNF-α concentration alone, but the TNF-α/IL-10 ratio could be a more adequate parameter for assessment of the inflammatory process in correspondence to genetic factors. Moreover, our observation also supports the hypothesis of epistasis between TNFA and IL10 genes in regulation of both TNF-α and IL-10.

**Comments on the studies of cytokines and AuAbs**

In paper VI, which was a cross-sectional study of different AuAbs we investigated anti-Jo-1 AuAbs (MSA), which were recorded in 23% of our PM/DM patients. A higher TNF-α/IL-10 ratio was observed in PM/DM patients with anti-Jo-1 antibodies than in those patients who were anti-Jo-1 negative. A tendency to higher TNF-α/IL-10 ratio was also observed in anti-Jo-1 positive patients who had ILD (ILD) compared to other groups, but there was no correlation with disease activity.

Among the different findings regarding AuAbs, cytokines and disease activities described in this thesis (paper I, II, IV and VI), the major findings were that serum levels IL-10 and TNF-α were increased in patients with PM/DM, MCTD and RA compared to controls, but not in patients with SLE. An association was determined between TNF-α and IL-10 and presence of AuAbs. A correlation was also found between disease activity and anti-U1 RNP levels, but not with anti-Ro or anti-La levels. No correlation between cytokine levels and disease activity in any of the investigated diseases was detected.
It is clear from the data presented in this thesis that serum levels of TNF-\(\alpha\) and IL-10 do not reflect overall disease activity or certain clinical phenotypes of individual organ systems in either MCTD, SLE, nor in PM/DM patients, but could merely reflect a mechanism underlying AuAb production.

The failure to correlate the results of the investigated cytokines with the disease activity as estimated by SLAM or other markers of disease activity such as CRP and total levels of immunoglobulins in papers I and II (MCTD and SLE patients) as well as by the physician’s global disease activity (VAS scale, paper VI, myositis patients) should, however, be seen in the light of the relatively small number of patients analyzed.

**Genetic studies (Papers III and V and VI)**

In order to achieve an increased understanding of the mechanisms that are involved in the production of TNF-\(\alpha\), IL-10 and AuAbs I chose to study genetic markers that could be of interest in this respect. Therefore, in papers III, V and VI, I investigated genes in both MHC and non-MHC regions in patients with MCTD and adult PM/DM.

**MHC genes**

We chose to examine the frequency of a SNP in the promoter region of the -308TNFA gene (G-308A TNFA) and two microsatellite markers, MHC class I-related gene, MICA, and TNFa in MCTD and/or PM/DM patients. HLA-DRB1 typing was performed to analyze an eventual association of MCTD with DRB1*04 (Dong et al. 1993) and myositis with DRB1*03 gene (Garlepp 1993). These four markers that we analyzed within the HLA region are spread over 1.2 Mbp on the short arm of chromosome 6. Additionally in MCTD we investigated HLA-DQA1 and −DQB1 genes.

Regulatory polymorphisms might be responsible for the observed phenotype of increased TNF-\(\alpha\) serum levels, since the TNF2 allele of the −308TNFA gene is associated with higher TNF-\(\alpha\) production in juvenile DM patients (Pachman et al. 2000). In MCTD no difference was observed between patients and controls in allele or genotype frequencies. Thus, it is likely that the increased serum levels of TNF-\(\alpha\) previously demonstrated in MCTD patients could be induced by environmental factors rather than genetics. Another possibility is that the patient group was too small to detect significant
associations or differences compared to healthy controls. Therefore, a role of TNF-α in the pathogenesis of MCTD could still not be excluded despite the fact that the gene polymorphism of this cytokine was not associated with MCTD.

In PM/DM we demonstrated that the TNF2 allele of the -308TNFA gene was associated with PM/DM. The increased frequency of the TNF2 allele of -308TNFA gene in PM/DM patients is consistent with a previous study in juvenile DM (Pachman et al. 2000) and also with other studies in autoimmune diseases (Hajeer et al. 2001). There was no difference between PM and DM subgroups.

Concerning the TNFa microsatellite polymorphism there were no statistical differences in the allele frequency between MCTD patients and controls (data not included) or between PM/DM patients and controls. Furthermore, no difference between PM and DM patients in frequency of TNFa microsatellite alleles was determined.

Our rationale to investigate MICA gene in our patients was that the expression of this highly polymorphic MICA molecule on the cell surface of fibroblasts and endothelial cells, could make this molecule a possible target for the recognition by specific antibodies and/or T cells during the immune response in MCTD and/or myositis. This molecule could thus play a role in the pathogenesis of MCTD and/or myositis.

Our investigation of the MICA gene in MCTD and PM/DM revealed different allele associations in each disease. In MCTD we demonstrated that the genotype of allele 5.1 of MICA gene in a homozygous state (MICA5.1-5.1) was more frequent than in controls. This was only significant before correction for multi-comparison, however. Our results also showed that the frequency of the allele 9.0 of the MICA gene (MICA9) was reduced in MCTD patients compared to controls. These data suggest that the MICA5.1 allele may be considered as a risk factor associated with susceptibility to MCTD and that the MICA9 allele may be considered protective for MCTD. In PM/DM we reported that the frequency of the MICA5.1 allele in a homozygous state (MICA5.1-5.1 genotype) was significantly increased in myositis patients compared to controls, even after correction.

To test the potential role of the DQ-DR haplotype in susceptibility to MCTD, we analyzed HLA-DRB1, DQA1 and DQB1 polymorphisms in our patients and controls. Our study demonstrated that HLA-DRB1*04 was more frequent in MCTD patients compared to healthy controls, but that the HLA-DRB1*01 frequency was not different in
MCTD compared to controls. However, our analysis revealed that the frequency of the shared epitope (SE) (DRB1*01 and DRB1*04) was significantly higher in MCTD patients compared to controls. Furthermore, we performed sub-typing of HLA-DR4 and −DR1 genes so that all MCTD patients who were DR4 and/or DR1 positive were sub-typed and the shared epitope alleles were confirmed (unpublished data). We further characterized those patients clinically to see if there are any correlation between the shared epitope and clinical phenotypes, as is the case in RA. We found that within our MCTD patients 9/24 patients had erosions (unpublished data). Furthermore, we found that 8/9 of MCTD patients who had erosions had shared epitope alleles. Regarding HLA-DQ we determined that DQ8 (DQA1*0301-DQB1*0302) was more frequent in MCTD patients than in controls. Our analysis of HLA-DRB1 genes in myositis patients confirmed earlier findings that the HLA-DR3 genotype is associated with PM/DM (Garlepp 1993).

A strong linkage disequilibrium of HLA-DRB1*03 allele (DR3) with the polymorphism at position −308 in the promoter region of the TNFA gene has been reported (Wilson et al. 1993). This linkage disequilibrium could be confirmed in our myositis studies. However, such a linkage has not been demonstrated for HLA-DRB1*04 gene, which was recorded in most of our MCTD patients (67%). This could possibly be a third possibility to explain the lack of association with the TNFA gene in our MCTD patients. To evaluate MHC associations in terms of haplotypes rather than individual alleles we aimed to reconstruct haplotypes for the analyzed markers. The presence of an extended haplotype, MICA4-TNF1-DRB1*04, is a separate risk factor for MCTD as it was found exclusively in MCTD patients compared to the controls.

In PM/DM patients we could demonstrate a positive association with the HLA-DRB1*03/TNF2 haplotype (ancestral haplotype). Extending this ancestral haplotype (AH 8.1) with MICA5.1 and TNFa microsatellite markers resulted in a “myositis-specific” haplotype with four markers (MICA5.1/TNFa2/TNF2/DRB1*03), which was significantly more frequent in patients compared to controls. In general, there was no difference between PM and DM subgroups regarding the frequencies of all investigated markers and there was no effect of gender on genetic risk/susceptibility to myositis.
The observation that MCTD associated with HLADRB1*04 confirmed a previous observation and demonstrated that MCTD associated-HLA alleles are distinct from SLE-, SSc-, PM/DM-associated alleles and supports the notion that MCTD is a distinct disease entity. The association with HLA-DRB1*04 in MCTD is shared with RA, but interestingly in MCTD patients the haplotypes with MICA were different from RA. In Spanish subjects the MICA allele 6 was found to be protective against shared epitope-associated RA (Martinez et al. 2001), while allele 6 of TNFa microsatellite was associated with susceptibility to RA (Martinez et al. 2000). These associations were not observed in our MCTD patients. In our MCTD study we did not have enough clinical material to prove whether genetic markers such as TNFA, TNFa, MICA HLA-DRB1 and HLA-DQA1 and −DQB1 independently contribute to MCTD. Nevertheless, from our interaction analysis in MCTD we could conclude that each of MICA5.1-5.1, MICA4 and DRB1*04 was associated with the disease.

To investigate the functional implications of our genetic association in PM/DM patients we analyzed serum levels of TNF-α in relation to TNFA genotype. In previous studies, TNF2 allele of −308 TNFA was reported to be associated with an increase in the expression of TNFA gene (Kroeger et al. 1996; Wilson et al. 1997). We could also confirm that PM/DM patients had higher serum levels of TNF-α compared to healthy controls. This effect could be explained by the relatively high frequency of TNF2 allele in myositis patients who were positive for the TNF2-DRB1*03 genotype. Higher levels of TNF-α were also observed in the group of patients who were positive for TNF1-DRB1*03 genotype. Thus, other factors could upregulate TNF-α production in TNF2 negative patients with myositis. In vitro studies have demonstrated that there were variations in inducibility of the TNFA gene in individuals with different HLA-DR types (Bendzen et al. 1988; Endres et al. 1988; Molvig et al. 1988).

To our knowledge this is the first study to demonstrate increased frequency of the component of ancestral haplotype, AH 8.1, (DRB1*03/TNF2) in adult patients with PM/DM. Additional genetic factors as well as environmental factors should be involved in the development of myositis.

Furthermore, the increased serum levels of TNF-α, as well as the increased frequencies of the TNFA gene and the TNFa2 microsatellite markers illustrates the
potential role of TNF-α molecule in the pathogenesis of myositis and based on our present material we can establish the functional importance of TNFA gene polymorphism in IIM. An increased frequency of the TNF2 allele was previously reported in juvenile DM and was in those patients associated with increased production of TNF-α in vitro as well as a marker of a more severe disease including long disease course and presence of pathologic calcifications (Pachman et al. 2000). Having shown these data we believed that further functional studies of the proinflammatory cytokine, TNF-α, in relation to clinical data and other genetic markers in myositis patients would clarify its role in the pathogenesis of myositis.

We can hypothesize that not only upregulation of TNF-α production, but misbalance between TNF-α and IL-10 production in patients with myositis may be one of the key factors in development of chronic inflammation accomplished with AuAb production. Thus, therapeutic intervention for normalization of the TNF-α/IL-10 imbalance might be beneficial for myositis patients.

In an effort to evaluate the role of cytokine gene polymorphism in development of ILD in myositis patients and despite encouraging preliminary results about association of certain genetic pattern with lung fibrosis (data not included), we were unable to show clear correspondence of the development of ILD with any of analyzed genetic markers. The relatively small number of observations limited further stratification of the groups for this analysis. Despite the rather limited number of patients included in our study, we could still detect significant genetic differences in cytokine polymorphisms in our myositis patient group compared to the controls. This reflects the importance of using carefully matched controls.

Non-MHC genes

In paper VI we investigated non-MHC genes, mainly G-1087A IL10 and G915C TGFBI (codon 25) polymorphisms in myositis patients. We did not determine any significant difference concerning allelic distribution or genotypes frequency for the IL10 or TGFBI alleles in our PM/DM patients compared to the controls. This could be due to the limited number of patients in our investigation. According to our data the C915 TGFBI allele is significantly more rare in DM patients in comparison with PM patients.
As this observation is the first for this marker it needs to be confirmed in other patient populations.

**GENERAL DISCUSSION**

Based on our aims to characterize immunological parameters and genetic markers in autoimmune diseases such as MCTD, SLE, RA and PM/DM patients we investigated the pattern of AuAb production in relation to cytokine pattern and their relation to other laboratory data and clinical phenotypes including disease activity and to some extent to genetic markers to understand if these parameters show differences or similarities between the disorders.

Our serial analysis of the AuAbs such as Ro/SSA and La/SSB and U1snRNP characteristic for SLE and MCTD, respectively, revealed both differences and similarities. Anti-U1snRNP AuAbs (68 KDa, RNP-A and RNP-C) and anti-Ro and anti-La AuAbs had a coordinated expression indicating that some AuAbs could be derived by the same mechanism. The main difference was the highly fluctuating anti-U1snRNP antibody levels, where these antibodies were high early in MCTD disease and then continuously decreased with time together with cytokines (TNF-α and IL-10) and disease activity, while in SLE anti-Ro and -La AuAb levels were fixed at an early stage of the clinically apparent disease and in most patients hardly changed with time. Due to the limits in quantity of patient’s sera we were unable to perform serial analyses of serum levels of TNF-α and IL-10 in patients with SLE to see if there was an association to AuAb production, as was the case for MCTD.

In patients with PM/DM we reported an association between the presence of anti-Jo-1 AuAb levels and high ratios of TNF-α/IL-10 serum levels. Thus, not only TNF-α serum levels, but the ratio between the serum levels of TNF-α and IL-10 could be important in the pathogenesis of PM/DM.

Based on our hypothesis that cytokine could have a genetic basis in chronic inflammatory diseases, I determined genes that could be relevant for cytokine production. A significantly higher frequency of markers from the ancestral, pro-inflammatory, haplotype (AH 8.1) in patients with PM and DM was detected. This proinflammatory haplotype includes two known markers from the ancestral haplotype AH 8.1 (DRB1*03
and TNF2), but also two other markers for the first time included by us, TNFa2 and MICA5.1. Our study supports a role of this proinflammatory haplotype as a factor in susceptibility to develop PM and DM. Moreover, our results further suggest that the HLA-DR3 positive haplotype rather than HLA-DR3 gene itself or other separate markers in the HLA region is an important factor in this aspect. Our data also demonstrated the possibility of inclusion of the TNFa2 microsatellite and MICA5.1 alleles in the extended ancestral haplotype 8.1.

Our study concerning cytokine gene polymorphisms is the first association study in adult PM/DM patients with cytokine genes as candidate genes. We determined an association between the TNF2 allele together with A–1087 IL10 allele in patients with PM and DM. Moreover, we determined an association between TNF2 allele and the ratios of TNF-α/IL-10 in serum, as well as with the appearance of certain AuAbs such as anti-Jo-1 AuAbs. Interestingly, the association between TNF2 allele encoding the high production of the proinflammatory cytokine and the IL10A allele encoding the low production of the anti-inflammatory cytokine revealed the synergistic effect we observed between the two polymorphisms, even though the TNFA and IL10 genes are on different chromosomes (chromosome 6 and 1, respectively) and that these genes will inevitably segregate independently. The synergistic effect between the two polymorphisms may move the balance towards higher serum levels of the proinflammatory cytokine (TNF-α) and aggravate the proinflammatory condition, which seems to be characteristic for PM/DM patients. Thus our data supports the hypothesis that certain cytokine gene polymorphisms could contribute to susceptibility of PM and DM as well as to the different phenotypic manifestations of disease, and that both TNF-α and IL-10 could be important factors in the misbalance of the immune system during these disorders.

Based on the results presented in our three MCTD studies we could conclude that MCTD patients share some distinct immunological properties with both RA and SLE and that MCTD might be considered as a separate disease entity according to these properties. Both cytokines and AuAbs could be considered as underlying pathogenic factors that maintain the abnormal immune response in MCTD patients. Furthermore, MCTD that is associated with HLA-DRB1*04 is genetically distinct from SLE, SSc, and PM/DM. HLA-associated alleles in MCTD were shared with RA, although RA and MCTD are
genetically distinct from each other regarding frequency of MICA and TNFa microsatellites. Further functional studies in relation to genetics could reveal more distinct differences between MCTD and RA.
CONCLUSIONS

From the studies presented in this thesis we can conclude that there is a difference between AuAb patterns with time in patients with MCTD compared to SLE. In patients with MCTD anti-U1 RNP antibody levels were high early in the disease and highly fluctuating and decreasing with time. In contrast, anti-Ro/SSA and anti-La/SSB in SLE were fixed early in disease course and hardly changed with time. There were increased serum levels of TNF-α and IL-10 in MCTD and PM/DM patients, however unrelated to disease activity in both disorders. The genetic studies in MCTD and PM/DM indicate that genetic factors could contribute to disease susceptibility and that cytokine production could be genetically determined in these disorders.

Our immunological studies in MCTD, SLE and PM/DM may provide a clue about underlying pathogenic immunological factors and the mechanisms driving these factors in these different but related autoimmune rheumatic diseases, where we showed that both AuAbs and cytokines are associated to some extent, and that the diverse AuAb pattern in these diseases could indicate that various mechanisms drive different AuAbs in different diseases. One of these mechanisms underlying AuAb production could be the imbalance between pro- and anti-inflammatory cytokines. Variations in cytokines and AuAb production may have genetic basis. Overall we observed remarkable differences in MCTD patients compared to RA, SLE and PM patients.

The genetic studies in MCTD and PM/DM also specified the frequency of susceptible alleles in these two disorders. Moreover, these genetic studies were the first to demonstrate cytokine gene polymorphisms in MCTD and adult PM/DM patients and to provide the basis for haplotype analysis for future association studies concerning both SNPs and haplotype frequency distributions among different populations with MCTD and PM/DM.
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