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The role of Ro52 autoantibodies in congenital heart block

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To Marco and Elin

ABSTRACT

The presence of B cells producing autoantibodies is a common feature of many autoimmune conditions. The pathogenic role of the autoantibodies is often unclear, although they often serve as diagnostic markers and may be used as prognostic tools in some diseases. This thesis addresses the production of anti-Ro52 antibodies in patients with Sjögren's syndrome, and their pathogenic effect in the fetus after transplacental transport during pregnancy.

The autoimmune inflammation in Sjögren's syndrome targets exocrine organs. Lymphocytic infiltrates develop in the salivary glands, and within these infiltrates germinal center-like structures may develop. In the initial studies of this thesis the molecular requirements for lymphocyte recruitment to the salivary glands, as well as morphological and functional properties of the germinal center-like structures were investigated. Chronically inflamed salivary glands exhibited an increased expression of adhesion molecules (VCAM-1, VLA-4, ICAM-1 and LFA-1), detected on endothelium and on infiltrating mononuclear cells. In addition, B and T cell-attracting chemokines (CXCL13, CCL21 and CXCL12) were expressed by epithelial cells in the glands, and the chemokine receptor (CXCR5) was detected on infiltrating lymphocytes. Networks of follicular dendritic cells were demonstrated throughout the large infiltrates, while autoantibody-producing plasma cells and apoptotic cells were mainly localized to the margins of these infiltrates. The presented studies provide a molecular basis for lymphocyte-recruitment to the target organ and demonstrate functional ectopic germinal centers with local autoantibody production in the target organ. These structures might actively promote chronic inflammation, and differences in the microenvironment and structure compared to ordinary germinal centers might contribute to a disturbance in the selection process allowing autoreactive clones to develop more frequently.

During pregnancy Ro52 antibodies are transferred to the fetus, which may lead to development of congenital heart block. Although rare, the condition is often fatal and the majority of live borns require a pacemaker at an early age. To elucidate the pathogenic importance of anti-Ro52 antibodies in the course of congenital heart block, their role was investigated *in vivo* in pregnant women and in a murine model for the disease, as well as *in vitro* in primary rat cardiomyocyte cultures. Seropositive pregnant women were prospectively followed and fetal progression monitored using Doppler echocardiography. One-third of the fetuses developed a transient AV block I, demonstrating that fetal affection is far more common than previously appreciated. The study also revealed that congenital heart block develops gradually, a finding essential for therapeutic decisions and understanding of the underlying pathogenic mechanism. Thorough epitope mapping of the humoral response in pregnant women with anti-Ro52 antibodies revealed a specific serologic marker associated with congenital heart block, a finding enabling risk assessment and increasing the chances for early detection of block and successful fetal outcome. The specific antibodies associated with block were cloned and demonstrated to bind to the cell surface of neonatal cardiomyocytes *in vitro*, dysregulating calcium homeostasis, leading to accumulating intracellular levels of calcium and eventually inducing apoptosis. The pathogenic effect of the antibodies was verified in a murine model of congenital heart block. In conclusion, our results have led to the proposal of a mechanism for development of congenital heart block in which specific maternal anti-Ro52 antibodies have a central pathogenic role through interaction with fetal cardiomyocytes, affecting their function and resulting in a permanent cardiac insult.

Every day is a gift

that is why it is called the present

LIST OF PUBLICATIONS

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

I. S Salomonsson, P Larsson, P Tengnér, E Mellquist, P Hjelmström, M Wahren-Herlenius. **Expression of B cell-attracting chemokine CXCL13 in the target organ and autoantibody production in ectopic lymphoid tissue in the chronic inflammatory disease Sjögren's syndrome.** 2002, Scandinavian Journal of Immunology 55:336-42.

II. S Salomonsson, M Jonsson, K Skarstein, KA Brokstad, P Hjelmström, M Wahren-Herlenius, R Jonsson. **Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome.** 2003, Arthritis and Rheumatism 48:3187-201.

III. S Salomonsson, M Wahren-Herlenius. **Local production of Ro/SSA and La/SSB autoantibodies in the target organ coincides with high levels of circulating antibodies in sera of patients with Sjögren's syndrome.** 2003, Scandinavian Journal of Rheumatology, 32:79-82.

IV. E Theander, A Brucato, S Gudmundsson, S Salomonsson, M Wahren-Herlenius, R Manthorpe. **Primary Sjögren's syndrome – treatment of fetal incomplete atrioventricular block with dexamethasone.** 2001, Journal of Rheumatology 28:373-6.

V. S Salomonsson, T Dörner, E Theander, K Bremme, P Larsson, M Wahren-Herlenius. **A serologic marker for fetal risk of congenital heart block.** 2002, Arthritis and Rheumatism 46:1233-41.

VI. L Horvath, S Salomonsson, SE Sonesson, K Bremme, M Wahren-Herlenius. **Ro/SSA and La/SSB autoantibody level variation before, during and after pregnancy in women with systemic rheumatic diseases giving birth to children with and without neonatal lupus.** Submitted for publication.

VII. SE Sonesson, S Salomonsson, LA Jacobsson, K Bremme, M Wahren-Herlenius. **Signs of first-degree heart block occur in one-third of fetuses of pregnant women with anti-SSA/Ro 52-kd antibodies.** 2004, Arthritis and Rheumatism 50:1253-61**.**

VIII. S Salomonsson, L Ottosson, P Säfsten, D Hof, H Brauner, M Sunnerhagen, J Raats, M Wahren-Herlenius**. Cloning and characterization of two human Ro52-specific monoclonal autoantibodies directed towards a domain associated with congenital heart block.** 2004, Journal of Autoimmunity 22:167-77.

IX. L Ottosson, S Salomonsson, J Hennig, SE Sonesson, T Dörner, J Raats, VK Kuchroo, M Sunnerhagen, M Wahren-Herlenius. **Structurally derived mutations define congenital heart block-related epitopes within the 200-239 amino acid stretch of the Ro52 protein.** Submitted for publication.

X. S Salomonsson, SE Sonesson, L Ottosson, S Muhallab, T Olsson, M Sunnerhagen, VK Kuchroo, P Thorén, E Herlenius, M Wahren-Herlenius. **Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis and mediate congenital heart block.** Submitted for publication.

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LIST OF ABBREVIATIONS

aa	amino acid
APC	antigen presenting cell
AQP	aquaporin
AV	atrioventricular
BCA-1	B cell attracting chemokine-1 (CXCL13)
CD	clusters of differentiation
CDR	complementary-determining region
CHB	congenital heart block
CSN	central nervous system
DA	Dark Agouti
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid
ECG	electrocardiography
ELISA	Enzyme Linked Immunosorbent Assay
FDC	follicular dendritic cell
GFP	green fluorescent protein
HEV	high endothelial venule
HLA	human leukocyte antigen
hY RNA	human cytoplasmic ribonucleic acid
ICAM-1	intracellular adhesion molecule-1 (CD54)
Ig	immunoglobulin
IL	interleukin
kD	kilo Dalton
LFA-1	lymphocyte function-associated antigen-1 (CD11a)
NLE	neonatal lupus erythematosus
mRNA	messenger ribonucleic acid
PBC	primary biliary cirrhosis
RA	rheumatoid arthritis
RBCC	RING/B box/coiled-coil
RNA	ribonucleic acid
RNP	ribonucleoprotein
SA	sinus atrial
SDF-1	stromal derived factor-1 (CXCL12)
SLC	secondary lymphoid tissue chemokine (CCL21)
SLE	systemic lupus erythematosus
SMAc	α-smooth muscle actin
SS	Sjögren’s syndrome
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TRIM	tripartite motif protein
UCTD	undifferentiated connective tissue diseases
VCAM-1	vascular cell adhesion molecule-1 (CD106)
VLA-4	very late activation antigen-4 (CD49d)

1 RATIONALE

Autoimmune disorders are thought to result from a loss of immunological self-tolerance, resulting in immune reactions directed against self and presence of autoreactive T cells and/or autoantibody-producing B cells.

This thesis focuses on the pathogenic role of autoantibodies directed against the intracellular antigen Ro52. Antibodies against this ubiquitously expressed protein serve as a serologic marker for patients with the rheumatic diseases Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE), and a direct pathogenic role for these autoantibodies has been proposed in babies born with the passively acquired autoimmune manifestation congenital heart block (CHB). By studying the antibodies against Ro52 in the target organ of patients with Sjögren's syndrome and the direct effects of the antibodies in the course of congenital heart block, we hope to contribute towards a better understanding of their role in these autoimmune conditions, eventually leading to a better defined mechanism of disease development and provide the basis for development of more specific therapies.

2 BACKGROUND

The immune system has evolved to defend the host against infection. The cells enacting immune defense originate from the bone marrow, where many of them mature. By tradition the immune response is divided into non-specific or innate and specific or adaptive immunity, which have several overlapping functions (Janeway et al 2001).

2.1 THE INNATE IMMUNE SYSTEM

The innate immunity provides a first line of defense against many common microorganisms. This immediate defense system includes physical barriers such as skin and mucosa as well as phagocytic cells recognizing common constituents expressed on the surface of invading pathogens. Recognition of pathogens by surface receptors on phagocytic cells triggers engulfment of the invader and induces secretion of cytokines that can lead to a local inflammatory reaction. Upon initiation of an inflammatory response effector cells are recruited to the site of infection resulting in increased permeability of the blood vessels, increased blood flow and leakage of fluid and circulating immune cells into surrounding tissues.

2.2 THE ADAPTIVE IMMUNE SYSTEM

The adaptive immunity is antigen-specific and can be divided into humoral immunity mediated by antibody-producing B cells, and cell-mediated immunity mediated by T cells.

Each B and T cell clone expresses a single type of receptor with a unique specificity. Upon interaction between its receptor and a specific antigen the cells are activated, resulting in cell proliferation and differentiation into effector cells. This process normally takes place in lymphoid organs which are constantly encountered by naïve B

and T cells and where antigens from surrounding tissue are presented by antigen presenting cells (APCs).

My thesis work has been focused on B cells and antibody-production and this process will be described in some detail.

2.2.1 B cell maturation in germinal centers

B cells develop in the bone marrow and mature into antibody-producing plasma cells in peripheral lymphoid organs. Antibodies, the product of a complex set of gene rearrangements, specific mutations and antigen-driven selection taking place during B cell maturation, are composed of two heavy and two light polypeptide chains. Each chain has a variable domain, including three loops of highly variable sequence, complementary-determining regions (CDRs), which largely determine the antigen specificity of the mature antibody.

Naive B cells traverse the lymphoid tissues and if encountering a specific antigen are activated by helper T cells and induced to mature into effector cells. This primary activation of B cells can initiate formation of germinal centers in the B cell areas within the lymphoid organs. In these highly organized structures B cells proliferate, go through positive and negative selection, and eventually differentiate into high-affinity plasma cells and memory B cells.

Germinal centers are composed of specialized microenvironments with certain morphological characteristics (see Figure 1). The rapidly proliferating B cells form the dark zone, which is surrounded by the light zone composed of follicular dendritic cells (FDCs), maturating B cells and T cells. The FDCs are professional APCs and form a dense network providing signals essential for B cell survival and further development (Liu et al 1996). They present native antigens and only the B cells expressing high affinity receptors can bind, be engaged in antigen-specific interactions with helper T cells and survive. The antibody affinity for the specific inducing antigen gradually increases during B cell maturation in germinal centers through a process called affinity maturation, involving somatic hypermutation of the immunoglobulin V-domain genes (Berek et al 1991). B cells failing to increase their antigen-affinity undergo apoptotic cell death and are phagocytosed (Takahashi et al 2001, van Eijk et al 2001). B cells that successfully bind antigen and survive selection pass through the mantle zone before leaving the germinal centers as either antibody-producing plasma cells or memory B cells. Plasma cells enter the circulation and are predominately located in the bone marrow, lymph nodes, spleen and mucosal tissues. The majority of the memory B cells reside in distinct areas of the secondary lymphoid organs, while some memory cells leave and migrate through the blood.

B cells enter the lymphoid tissue by crossing high endothelium venules (HEVs), in a specific and efficient process largely governed by interaction between complementary adhesion molecules on endothelia and B cells (Girard et al 1995, Butcher et al 1996). The most important adhesion molecules expressed in lymphoid organs are selectins (of E, L and P-type), integrins (e.g. LFA-1 and VLA-4) and immunoglobulins ICAM-1 and VCAM-1. Synthesis, expression and avidity of these adhesion molecules are in turn regulated by chemical mediators, particularly chemokines (Campbell et al 2000, Mebius 2003). Chemokines are small, mostly secreted proteins and are classified into four families (C, CC, CXC and CX3C) based on the number and spacing of cysteine residues in the amino-terminal part of the molecules (reviewed in Ansel et al 2001). In addition to inducing expression of adhesion molecules, chemokines are active in promoting cell migration and retention in lymphoid tissue, having an essential role in the development and organization of lymphoid organs. Examples of chemokines of importance for a

functioning lymphoid organ are the T cell attracting CCL21 (SLC), the B cell attracting CXCL13 (BCA-1) and the cell pro-retention chemokine CXCL12 (SDF) (Cyster 1999, Hasegawa 2001).

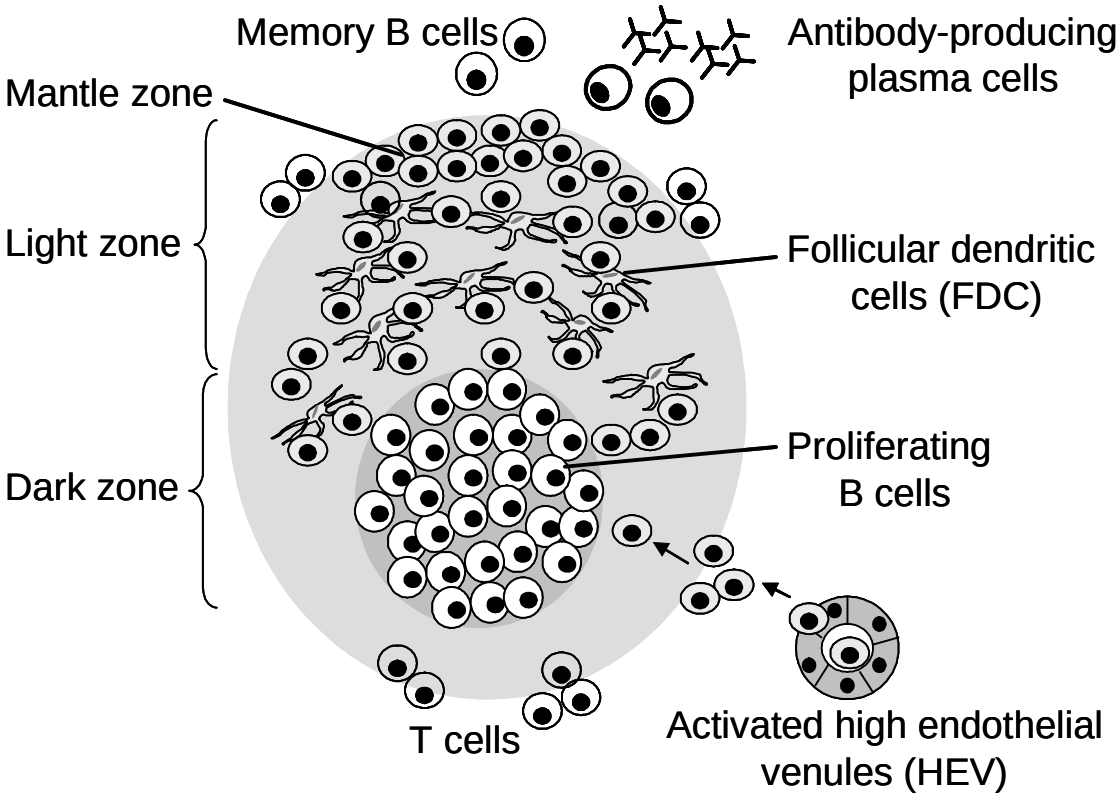

Figure 1. Germinal centers are highly specialized microenvironments in which B cells proliferate, are selected for antigen binding and mature. B cells enter the lymphoid organ through activated HEVs. Activated B cells are initiated to proliferate, forming densely packed cell clusters called "dark zone". As B cells mature they stop dividing and enter the "light zone", where they interact with the FDCs and helper T cells. During development into effector cells B cells undergo somatic hypermutation and are selected for antigen binding, resulting in highly specific plasma and memory cells.

2.3 AUTOIMMUNITY

Autoimmune diseases are chronic conditions characterized by a loss of immunological tolerance to self-antigens, resulting in activation of autoreactive T and/or B cells. Collectively, autoimmune diseases are estimated to affect 4-5% of the population (Wandstrat et al 2001), and females generally have a higher disease incidence compared to males (Whitacre 2001).

How tolerance is lost, favoring expansion of autoreactive T and B cells, is not completely understood, although it is clearly very complex and likely to involve both environmental and genetic factors. Mechanisms involving bacterial and viral infections, hormonal factors, ultraviolet light, or stress, leading to exposure of normally hidden antigens or modifications of self-antigens rendering them autoantigenic, have all been suggested. In a recent report neutrophils are presented as potent participants in induction of autoimmune disease, in a scenario in which these cells might expose intracellular

antigens, mediating a first contact between hidden antigens and immune cells (Brinkmann et al 2004).

The genetic mechanisms contributing to development of autoimmunity are intensively explored. HLA-linkage has been shown in most autoimmune diseases, which could be explained by different alleles having different abilities to present peptides to autoreactive T and B cells (Marrack et al 2001). However, the current opinion is that the large majority of autoimmune diseases display a complex inheritance pattern, in which additional non-HLA genes are of great influence in predisposing for disease.

There are autoimmune diseases specific for nearly every organ in the body, and in many cases the response is targeted to an antigen specifically expressed in that organ. Insulin-dependent diabetes (pancreatic β islets), multiple sclerosis (CNS) and Grave's disease (thyroid gland) are examples of "organ-specific" disorders. In other autoimmune diseases such as SLE, the response is not directed towards one single cell type but antigens widely expressed throughout the host are targeted. However, there are intermediates involving specific organ(s) which might have an additional systemic involvement, as evident in rheumatoid arthritis (RA) affecting the joints and in SS in which exocrine organs are the main targets.

2.3.1 B cells in autoimmunity

The presence of B cells producing autoantibodies is one of the characteristic features of many autoimmune conditions. Autoantibodies often serve as diagnostic markers and can in some instances also be used as a prognostic tool, as for anti-DNA antibodies in SLE (Tan 1991) and antibodies against anti-glutamic acid decarboxylase and islet cell antigen-2 in insulin-dependent diabetes (Lernmark 1999). However, autoantibodies are not restricted to autoimmune patients alone; poly-reactive antibodies binding to self-structures with low affinity can also be detected in healthy individuals (Dighiero et al 1999).

How functionally silenced autoreactive B cells present in the circulation can be induced and stimulated to autoantibody production is not clear. Several autoimmune disorders are characterized by an abnormal B cell repertoire, such as in SLE, RA, SS and autoimmune thrombocytopenia**.** Studies of animal models and successful clinical trials directed to eliminate B cells in certain autoimmune conditions support involvement of defects in pathways influencing B cell survival or activation (Tuscano et al 2003). Apoptosis, a pathway for eliminating redundant, damaged or infected cells, occurs in embryogenesis, immune responses and carcinogenesis (Reed 2000). Furthermore, this pathway for programmed cell death has a regulatory role in maintaining lymphocyte homeostasis by deletion of autoreactive B and T cells, and defects in this process have been considered to play a role in the pathogenesis of several autoimmune diseases (Chervonsky 1999). However, considerable evidence has appeared favoring a scenario in which pathological autoantibodies result from an antigen-driven selection of autoreactive B cells, including somatic mutations (Shlomchik et al 1987, Stott et al 1998, and reviewed in Link et al 2002).

Although the exact role of autoantibodies in relation to disease initiation and/or propagation is not established, a direct pathogenic role is demonstrated in patients with anti-DNA antibodies and clinically findings of nephritis (Su et al 2003). An increased understanding of the mechanism of how autoantibodies are associated to disease activity and clinical symptoms would be of great interest and probably help in understanding the mechanism behind these disorders, and thereby enable development of more specific therapies for these conditions.

2.3.2 Sjögren's syndrome

Sjögren's syndrome (SS) is a chronic inflammatory disorder characterized by a progressive destruction of the exocrine glands, primarily the salivary and lacrimal glands (Jonsson et al 2001). The autoimmune exocrinopathy includes progressive mononuclear cell infiltration of affected tissues leading to oral and ocular dryness (sicca syndrome). In addition, affected individuals may suffer from chronic fatigue, arthralgia, and manifestations affecting musculoskeletal, pulmonary, gastric, hematologic, dermatologic, renal and neurological systems. When the symptoms occur alone the condition is termed primary Sjögren's syndrome (pSS), while if associated with another autoimmune disease, the condition is termed secondary Sjögren's syndrome (sSS).

B cells are thought to play an important role in SS, and disturbances in the peripheral B cell population of SS patients have been demonstrated (Bohnhorst et al 2001). Furthermore, patients with SS have an increased risk of developing B cell lymphomas, reflecting both B cell activation and loss of B cell tolerance. The clinically most important and best characterized autoantibodies in SS are directed against the intracellular proteins Ro/SSA and/or La/SSB, which can be detected in 60-90% of patients (Wahren-Herlenius et al 1999).

The prevalence for SS is 0.1-3% in general population, depending on the classification criteria used (Fox et al 1986a, Jacobsson et al 1989, Bjerrum 1997, Dafni et al 1997). This autoimmune condition predominantly affects women, with a female to male ratio of 9 to 1, and may appear at any age but most often develops between 30-50 years of age.

2.3.2.1 Diagnostic criteria

There are no universally accepted classification criteria for SS, which complicates comparison of studies performed using different sets of criteria. The criteria most frequently applied are the Copenhagen criteria (Manthorpe et al 1986), the Californian criteria (Fox et al 1986b) and the European criteria (Vitali et al 1993), which were recently revised by the European-American Consensus Group (Vitali et al 2002). In this latter version both clinical and immunological involvement is considered and at least four of the following six criteria have to be met; I) ocular symptoms, II) oral symptoms, III) ocular signs, IV) focal infiltrates in minor salivary glands, V) salivary gland involvement and VI) presence of autoantibodies against Ro and/or La. Of these six criteria the glandular histopathology (IV) and/or a positive serology (VI) have to be fulfilled in order to fulfill these classification criteria for SS. See Table 1 for classification criteria for patients in the present studies.

2.3.2.2 Involvement of salivary glands

One of the classification criteria for SS is lymphocytic infiltrates of the salivary glands. The mononuclear infiltrates consist mainly of $CD4^+$ T cells, while 5-15% are B cells (Fox et al 1984). Infiltrates develop in periductual and acinar areas, and when becoming extensive replace large portions of the glands. Most T cell receptors (TCR) are of α/β phenotype and the TCR V family repertoire has been shown to be limited to an oligoclonal expansion of certain T cells, suggesting an antigen-driven induction of these cells (reviewed in Sumida et al 1997). The B cells and local production of autoantibodies observed in infiltrates within salivary glands of patients with SS will be discussed in detail in relation to the work included in this thesis in the *Results and Discussion*.

Patients with SS have a 40-fold increased risk of developing lymphomas compared to the general population, and B cell non-Hodgkin's lymphoma is a serious complication

associated with disease (Kassan et al 1978, and reviewed in Mariette 1999). The lymphomas are usually low grade marginal zone lymphomas arising in the salivary glands, but might transform into a high grade lymphoma (Voulgarelis et al 1999).

Alterations in the expression pattern of immune mediators such as cytokines and chemokines have been suggested to be involved in the mechanism behind induction and maintenance of the immunological dysregulation apparent in the salivary glands in SS. The massive infiltration of inflammatory cells in the glands result in structural damage leading to disturbed function, a phenomena also seen in the target organ in other autoimmune diseases. Different immune mediators have been studied in SS, and several cytokines, chemokines and adhesion molecules have been found in increased amounts in salivary glands of patients. The chemokines are thought to induce expression of adhesion molecules mediating adhesion and migration of inflammatory cells to the glands. The elevated levels of chemokine expression evident by the epithelial and infiltrating cells in the glands of SS patients may be induced directly or secondary to change in cytokine levels.

Disturbances in apoptosis have been suggested to play a role in SS (reviewed in Manganelli et al 2003). Apoptosis of ductual and acinar epithelial cells in minor salivary glands of SS patients occurs, possibly resulting in impaired function. However, reports of increased apoptosis (Kong et al 1997) have been somewhat contradicted by others finding no difference between SS patients and controls (Ohlsson et al 2001). Furthermore, a low incidence of apoptosis in infiltrates has been demonstrated, possibly leading to marked, chronic accumulation of cells contributing to chronic inflammatory response in SS (Kong et al 1998, Ohlsson et al 2001). Apoptotic studies on salivary glands in SS are thus contradictory and how this process may be involved in the glandular destruction remains unclear. During proteolytic cleavage taking place in the apoptotic pathway previously hidden determinants may be expressed on the cell surface and proteins of a diverse subcellular distribution may be clustered together with apparently unrelated intracellular components. This could result in epitope-spreading, a phenomena that has been observed in the autoantibody response against Ro60, Ro52 and La in animal studies (Topfer et al 1995, Keech et al 1996, Scofield et al 1996, Tseng et al 1997), as well as in a the Ro60 response in a patient with SS (Wahren et al 1998).

The discovery of aquaporins initiated a new possible target in the course of salivary gland dysfunction seen in patients with SS. The diminished secretion in salivary glands of SS patients has been suggested to depend on decreased expression of aquaporin (AQP)-5, a water-specific membrane channel protein present on acinar cells (Steinfeld et al 2001). The role of AQP-5 in pathogenesis is controversial, and others have demonstrated an equal density and distribution of these proteins in SS patients and controls (Beroukas et al 2001). Furthermore, another isoform, AQP-1, present in the myoepithelial cells surrounding the acini is significantly down-regulated in SS patients (Beroukas et al 2002).

Onset of SS and many other autoimmune conditions are notably more prevalent in women and disease onset often coincides with stages in life characterized by great hormonal changes, such as during puberty, child-bearing age or menopause (Parke 2000). Sex hormones have naturally been suggested to be responsible for the increased incidences of autoimmune diseases, and the role of estrogen has been extensively studied although the pathway by which it might increase the immune responsiveness and be involved in the course of disease remains to be elucidated. Observations that estrogen can induce surface expression of Ro/La antigen in a dose-dependent manner *in vitro* (Zhang et al 2000) suggest a possible role in the initial course of disease, although the clinical relevance of these results in relation to findings that SS occurs more frequently in postmenopausal women are difficult to explain. Furthermore, animal models for SS demonstrate beneficial effects from androgens by reducing glandular inflammation (Sullivan 1997).

2.3.3 Ro/La autoantigens

Ro and La autoantigens are ubiquitous in all investigated nucleated cells, both in humans and mammals (Lee et al 1985, Byers et al 1990, Chan et al 1991, Shusta et al 2003). They are intracellular proteins and are thought to associate with one of four hY RNAs (1, 3-5), forming a cytoplasmic ribonucleoprotein (RNP) complex (Boire et al 1990, Peek et al 1993, Fabini et al 2000). The hY RNAs range from 83 to 112 nucleotides in length and they all form a similar stem-loop structure. A schematic drawing of the Ro/La RNP complex is presented in Figure 2. Lately, several other proteins have been suggested to associate with the complex (Bouffard et al 2000, Fabini et al 2000).

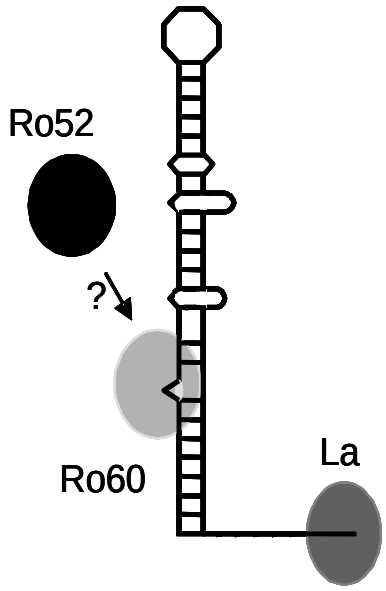

Figure 2. Schematic drawing of the Ro/La RNP complex. The hYRNA forms a stem-loop structure to which Ro60 and La bind. Ro52 possibly associates with the complex by protein-protein interactions with Ro60.

The La protein associates to the uridine rich stretch at the 3' terminal of the hY RNA. La can shuttle between the cell nucleus and cytoplasm but predominantly resides in the nucleus (Simons et al 1994, Keech et al 1995, Ohlsson et al 2002). Binding of the La protein to RNA has been demonstrated to protect from cleavage, suggesting a role in transcription termination (Wolin et al 2002). Furthermore, La can bind to several viral RNAs and is essential for the replication of these viruses. The capacity of binding to viral RNA has been proposed to be involved in inducing the immune reaction against the intracellular RNP complex. A proposed mechanism involves association of La-viral RNA leading to an immune reaction against the La protein possibly followed by epitope-spreading within the RNP complex, a phenomena that is supported by experiments in mice (Topfer et al 1995, Tseng et al 1997). Furthermore, studies in transgenic mouse models suggest that the loss of B cell tolerance to La is in large due to failure to activate T cells (Aplin et al 2003).

The Ro/SSA antigen consists of two distinct proteins, Ro60 and Ro52, which share no homology. The Ro60 protein (reviewed in Chen et al 2004) is expressed both the in cytoplasm and the nucleus (Peek et al 1993, Simons et al 1994, Keech et al 1995, Ohlsson et al 2002), and associates to the stem of the hYRNA via a conserved region containing a bulged cytidine on the hY RNA stem-loop (Wolin et al 1984, Pruijn et al 1991). The Ro60 protein has been implicated to have a role in the discard pathway of defect 5S RNA (O'Brien et al 1994, Labbe et al 1999). Mice lacking Ro60 were found to develop lupus-like symptoms including production of autoantibodies, and a mechanism including expression of misfolded ribosomes initiating the autoimmune reaction was proposed (Xue et al 2003). Ro52 has been suggested to associate with the Ro/La RNP complex, possibly

by protein-protein interactions with Ro60, as indicated by several reports (Ben-Chetrit et al 1988, Pruijn et al 1991, Slobbe et al 1992). Besides precipitation assays in which antibodies against Ro60 bind Ro52 (Fabini et al 2000) there are reports of epitope-spreading to Ro52 in animals immunized with Ro60 or La (Keech et al 1996, Tseng et al 1997). There are weaknesses in these studies, such as using patient sera in precipitation and antigenic spreading in animals could possibly by a result of cross-reactivity rather that intermolecular spreading. There are also conflicting reports where no association between Ro60 and Ro52 proteins could be demonstrated (Boire et al 1990, Kelekar et al 1994). More data has to be collected to finally define the relation between Ro52 and the remaining part of the RoRNP complex. Due to the central role that Ro52 has in this thesis it will now be described in some detail.

2.3.3.1 Ro52 protein

The Ro52 protein is a 475 amino acid (aa) residue protein and belongs to a large family of related RING/B box/coiled-coil (RBCC) or tripartite motif proteins (TRIMs) (Reymond et al 2001), see Figure 3. The TRIM proteins share the same domain structure with two N-terminal zinc-fingers and a coiled-coil, and the aa identity is 40-50%. Ro52, or TRIM21, is localized on human chromosome 11p15, as are several other RBCC-B30.2 proteins.

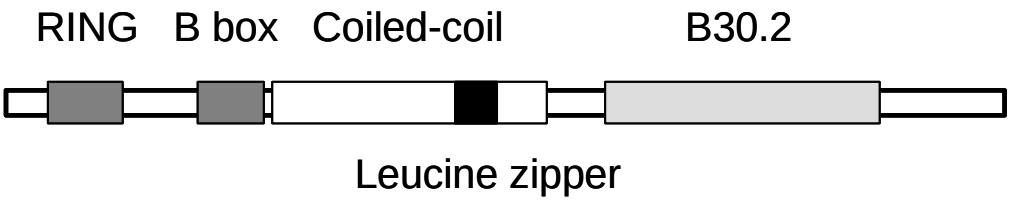

Figure 3. A schematic view of the Ro52 autoantigen, including the zinc fingers RING and B box (illustrated as grey boxes), the coiled-coil (white) encompassing the predicted leucine zipper (black) and the C-terminal B30.2 domain (light grey).

The RING finger and B box are zinc finger motifs. Their exact role in Ro52 is not defined but generally these domains are of importance in protein-protein interactions, and RING fingers often occur in ubiquinating enzymes (Joazeiro et al 2000). The coiled-coil region includes a predicted leucine zipper comprising aa residues 211-232. Leucine zippers form an α-helical structure where every seventh aa residue consists of a leucine. Each turn in the α-helix is comprised of 3.6 aa residues and a leucine appears every second turn facing the same direction and is often involved in protein-protein interactions (Paris et al 2003). Accordingly, Ro52 has been reported in dimer-formation (Wang et al 2001). The C-terminal part of Ro52 has high similarity to the B30.2 domain, which is not yet associated with a defined function.

The subcellular distribution of Ro52 has been studied using several techniques, detecting protein with affinity-purified patient sera and more recently by fusing the Ro52 protein with green fluorescent protein (GFP). Ro52 can be found in both the nucleus and cytoplasm, although more abundantly expressed in the cytoplasm, and has been suggested to participate in nuclear/cytoplasmic shuttling (Simons et al 1994, Keech et al 1995, Pourmand et al 1998a, Ohlsson et al 2002). Subcellular distribution of deletion clones of Ro52 proposes a role in cytoplasm-retention for the central coiled-coil domain of the protein (Pourmand et al 1998a).

Ro52 is an intracellular protein and the pathway by which this protein is exposed to the immune system and tolerance subsequently lost, allowing production of autoantibodies is puzzling, although several mechanisms have been suggested. Cell stress, mediated by ultraviolet irradiation or heat-shock in cultured human keratinocytes, has been shown to induce cell surface expression of Ro52 (Zhang et al 2000, Saegusa et al 2002). This has been related to the clinical findings of light-sensitivity exhibited by some patients with Ro and La autoantibodies. Apoptosis is also considered a possible mechanism for exposing intracellular antigens, and GFP-coupled Ro52 has been demonstrated on the surface of apoptotic cells in a human epithelial cell line (Ohlsson et al 2002). An additional potential factor is the role of hormones in inducing Ro52 expression. Estradiol can induce expression of Ro52 in human cell lines (Wang et al 1996, Sakabe et al 1998, Zhang et al 2000), and 17-β-estradiol has been shown to have a synergistic effect on Ro52 expression together with ultra violet irradiation in cultured human keratinocytes (Jones 1992).

In addition to full-length Ro52 an alternatively spliced transcript has been described, Ro52β. This isoform lacks aa residue 168-245 encoding the predicated leucine zipper (Chan et al 1995). Ro52β mRNA has been detected in several tissues, e.g. fetal and adult heart, lung, kidney, brain, as well as in salivary glands (Chan et al 1995, Buyon et al 1997b, Bolstad et al 2003), but no protein expression of this truncated form has been demonstrated *in vivo*.

The TRIMs are well conserved throughout evolution, indicating a functional relevance of this specific arrangement. Furthermore, they are often associated with transcriptional regulation, and due to sequence similarities the role of Ro52 in protein dimer formation and in transcriptional activity has been examined (Wang et al 2001). Several TRIM proteins have been demonstrating participating in the ubiquitination pathway (Lorick et al 1999), a tightly regulated process for enzymatic degradation of intracellular proteins. Ubiquitin gets activated and is added to proteins to be destroyed, the subsequent degradation taking place in proteosomes. The exact role of Ro52 in this process remains to be elucidated, but findings of interaction with the deubiquinating enzyme UnpEL support its involvement (Di Donato et al 2001). Furthermore, mono-ubiquitination of Ro52 has been detected in cells (Fukuda-Kamitani et al 2002).

Another interaction suggested for Ro52, besides being associated with the Ro/La RNP complex, is binding to immunoglobulin G (IgG) heavy chain via the B30.2 domain (Yang et al 1999, Rhodes et al 2002). Calreticulin has been proposed to associate with the RoRNP complex and to interact with Ro52 (Cheng et al 1996), a theory supported by a spreading in antibody response to calreticulin in mice immunized with Ro52 (Kinoshita et al 1998).

2.3.4 Autoantibodies against Ro and La

Autoantibodies against the intracellular proteins Ro52, Ro60 and La are strongly linked to the chronic inflammatory conditions SS and SLE, but can also occur in other autoimmune conditions including RA, myositis, primary biliary cirrhosis (PBC)**,** as well as in asymptomatic individuals. The exact role of antibodies against Ro and La in disease pathogenesis is not fully understood, although a pathogenic role has been implicated in congenital heart block (CHB). CHB is a passively acquired autoimmune manifestation affecting the fetus in pregnant Ro/La seropositive woman, a condition that will be described in detail in following sections.

The specificity of the autoantibody response to Ro and La has been extensively studied using several different techniques, and dominant epitopes have been identified for all three autoantigens (reviewed in Wahren-Herlenius et al 1999 and Scofield et al 1999).

In an attempt to define the antibody profile against the Ro52 protein several groups have demonstrated an epitope within the predicted leucine zipper in the central part of the protein (Blange et al 1994, Buyon et al 1994a, McCauliffe et al 1994, Kato et al 1995, Dörner et al 1996). Additionally, antigenicity has been demonstrated to the N-terminal region of the Ro52 protein containing the zinc-finger domains (Buyon et al 1994a, Pourmand et al 1998b). Interestingly, many intracellular autoantigens are involved in essential cellular functions and functional domains within the proteins are often found autoantigenic (Tan 1989).

2.4 CONGENITAL HEART BLOCK

Neonatal lupus erythematosus (NLE) is a passively acquired autoimmune condition closely associated with maternal Ro/La autoantibodies (reviewed in Dörner et al 2000). The syndrome includes several clinical manifestations. Congenital heart block (CHB) and cutaneous lupus are the most common, while complications such as liver dysfunction and hematological disorders also occur, but less frequently. To date, CHB is considered a permanent condition, while the non-cardiac manifestations of NLE are transient and resolve as maternal antibodies are cleared from the neonatal circulation. CHB is potentially lethal and the fetuses are identified with conduction abnormalities in a structurally normal heart (reviewed in Buyon et al 2003b).

2.4.1 Atrioventricular (AV) block

CHB is a conduction abnormality affecting the atrioventricular (AV) node, resulting in a delay or block in the signal conduction (John et al 2004). It has been suggested that AV block is initiated as a first-degree block, which could progress through stages, where a second-degree follows and eventually an irreversible complete block (third-degree) might develop. An AV block I is due to an abnormal delay in conduction through the AV node resulting in a prolonged PR interval on ECG (Figure 4). A second-degree block is an intermediate failure of the AV conduction and may occur in different forms where most, but not all, atrial impulses are conducted to the ventricles. A third degree block is complete and no signals are conducted via the AV node, related with a low ventricular rate usually ranging from 40 to 60 beats per minute.

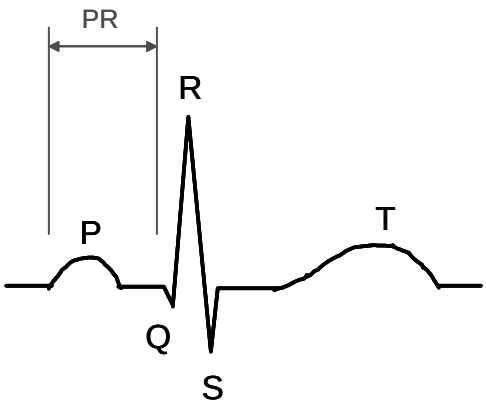

Figure 4. An ECG illustration of the P, Q, R, S and T waves. P waves are caused by atrial depolarization and corresponds to electrical impulses. Atrial contraction begins at about the middle of the P wave and continues during the PR segment (during which the impulse travel from the AV node through the conduction tissue).

2.4.2 Incidence of congenital heart block

CHB is a rare disease in the general population, with an incidence in newborn babies of 1/20 000 (Michaelsson et al 1972). The prevalence of a having a child with a third-degree block is about 2% in women known to have anti-Ro antibodies (Brucato et al 2001, Buyon et al 2001), and 10-20% in mothers with a previously affected infant (Waltuck et al 1994, Buyon et al 1998, Julkunen et al 2001b, Solomon et al 2003).

Fetal outcome in Ro/La positive women is hard to predict since CHB develops independently of maternal health status (Julkunen et al 1993, Press et al 1996). Furthermore, many mothers of affected children are completely asymptomatic and positive serology is not discovered until the fetus is found to be affected by heart block, which makes prospective identification nearly impossible.

2.4.3 Outcome in affected children and mothers

CHB is normally detected within 18-24 weeks of gestation (Buyon et al 1998), and is a life-threatening condition in which up to 30% of affected fetuses die (Waltuck et al 1994). Morbidity is substantial, with over two-thirds of affected children requiring permanent pacemaker implantation (Waltuck et al 1994, Buyon et al 1998, Eronen et al 2000).

Follow-up studies of children born with AV block include symptoms as fatigue, dizziness, effort dyspnea, low ventricular rate and a relatively high risk of sudden death due to cardiac failure (Esscher et al 1979, Michaelsson et al 1995). Recently, evidence has emerged that a subset of patients with CHB may develop dilated cardiomyopathy (DCM)(Eronen et al 2000, Moak et al 2001, Udink ten Cate et al 2001). The risk for developing DCM varies between studies, ranging from 6% in a retrospective study of 149 CHB patients (Udink ten Cate et al 2001) to 23% in a Finnish study evaluating 91 CHB patients (Eronen et al 2000). In addition, children born with CHB have been suggested to have a higher risk of developing connective tissue disorders later in life, SLE in particular (Hubscher et al 1997, Feist et al 2003), which has been contradicted in several large follow-up studies demonstrating no increased risk for children with CHB of developing rheumatic diseases (Buyon et al 1998, Eronen et al 2000, Martin et al 2002). The reported cases of developed SLE in children with CHB might be linked to a genetic predisposition for autoimmunity inherited from their autoimmune mothers rather than being linked to the child's own CHB.

A proportion of the mothers having affected children are asymptomatic at delivery but develop an autoimmune disease later in life, most commonly SS or SLE (McCune et al 1987, Waltuck et al 1994, Julkunen et al 2001a, Julkunen et al 2004). Despite exposure to identical circulating autoantibodies capable of mediating cardiac insult to the fetus, only a few cases with first-degree block have been described in the mothers (Dörner et al 1993).

2.4.4 Autoantibodies in congenital heart block

CHB is considered a passively acquired condition presumably arising from active transplacental transport of maternal autoantibodies against Ro and La entering the fetal circulation and potentially affecting the fetus. Although an association between CHB and maternal autoantibodies is well established, the direct effect is unclear.

Antibodies to Ro and La can be detected in nearly all mothers and their affected children, and several systematic analyses have been undertaken to identify the subpopulation and specificity of the maternal anti-Ro and -La antibodies that cause

disease. However, the cumulative data is somewhat heterogeneous and the linkage between CHB and antibodies to the Ro52, Ro60 and La proteins varies according to the laboratory method employed, which display different sensitivity and specificity. Despite these difficulties a majority of the attempts to define a specific maternal antibody profile have demonstrated an almost universal presence of antibodies targeting the Ro52 autoantigen (reviewed in Brucato et al 2003). This is supported by the clinical observations that CHB is more closely associated with maternal SS than with SLE, which more commonly display antibodies against Ro52 (Brucato et al 2001, Julkunen et al 2001a, Julkunen et al 2004). The efforts of describing a specific antibody profile associated with CHB will be further addressed in the *Result and Discussion* section.

CHB is a relatively rare condition and the recurrence rate is estimated to be 10-20% in pregnancies of mothers with a previous affected infant. From these observations it was suggested that development of CHB might depend on selective transfer of maternal antibodies into the fetal circulation. This was investigated in early serological studies comparing antibody profiles in mothers and their CHB children, demonstrating no significant selectivity in the antibody transfer and that antibodies specific for Ro52, Ro60 and La, as well as for all four IgG-antibody subclasses efficiently cross the placenta (Buyon et al 1994b, Tseng et al 1996).

The association between antibodies against Ro/La and cardiac conduction abnormalities has also been explored in pups after immunizing mothers with the recombinant proteins both in mice and rabbits (Miranda-Carus et al 1998, Boutjdir et al 1997, Xiao et al 2001b), and in passively injection of pregnant mice with human anti-Ro/La antibodies (Mazel et al 1999).

Antibodies against the alternatively spliced transcript Ro52β have been implicated in CHB. Ro52β mRNA was found at higher levels than full-length Ro52 mRNA in fetal heart during week 14 and 16 of gestation, preceding the time when heart block is first detected in the fetus (Buyon et al 1997a). Native Ro52β protein expression has never been demonstrated in animals or humans, although *in vitro*-translated 52β was shown to be antigenic using sera from Ro52 positive patients and from healthy donors (Chan et al 1995). A potential pathogenic effect of antibodies raised against Ro52β has been demonstrated in a murine model in which immunization with recombinant Ro52β protein could induce block in mice (Miranda-Carus et al 1998).

In addition to antibodies directed to the Ro and La protein, antibodies against the muscarinic acetylcholine receptor (Borda et al 2001), calreticulin (Orth et al 1996), p57 (Maddison et al 1995) and α-fodrin (Miyagawa et al 1998) have been suggested as markers or additional risk factors for CHB. However, no antibodies have been closer associated with CHB than anti-Ro52 antibodies, which are detectable in the majority of CHB mothers. However, considering clinical observations of only 10-20% reoccurrence rate in Ro/La positive mothers with a previously affected infant indicates that maternal autoantibodies are necessary but not sufficient for induction of disease.

2.4.5 Immunopathogenicity

CHB is thought to result from an inflammation of the fetal heart tissue mainly affecting the AV node, as documented in several histological studies of heart tissue from fetuses dying from CHB. Findings of lower anti-Ro antibody titers in a baby affected with CHB compared with his unaffected twin led to a hypothesis of selective deposition of anti-Ro antibodies in affected hearts (Harley et al 1985), and enrichment of anti-Ro antibodies in cardiac tissue from fetuses dying from CHB support this notion (Reichlin et al 1994). Histological studies have confirmed deposition of antibodies in CHB cardiac tissue (Litsey et al 1985, Lee et al 1987). Additional signs of inflammation in the fetal

heart including deposition of complement components, lymphocytic infiltrates, calcification and fibrosis have been reported in fetuses dying from CHB (Litsey et al 1985, Ho et al 1986, Lee et al 1987, Clancy et al 2004).

A complete AV block III is permanent and is thought to be a result of fibrotic scarring replacing large proportions of the AV node in particular (Lee 1990, Meckler et al 1998, Piercecchi-Marti et al 2003). Macrophages have been proposed to have a major role in the sustained inflammatory reaction and fibrotic process. They might act as phagocytes initially, engulfing cells as a part of the natural process in development of the fetal heart, but then shift from clearance to a state of hypersecretion of proinflammatory cytokines. This shift could possibly be promoted by binding of maternal autoantibodies to fetal cardiac cells, contributing to an increased and/or sustained inflammatory process. An increased secretion of the proinflammatory cytokine TNFα after stimulation with apoptotic human cardiomyocytes opsonized with anti-Ro and La sera has been demonstrated (Miranda-Carus et al 2000). Additionally, the macrophages have been suggested to increase calcification in the tissue by secreting alkaline phosphatase (Tintut et al 2002). Furthermore, macrophages might have a role in the subsequent fibrosis seen in complete AV block by secreting TGFβ. This is a pro-fibrotic cytokine that might contribute to transdifferentiation of cardiac fibroblasts into a scarring phenotype of myofibroblasts expressing α-smooth muscle actin (SMAc). An increased TGFβ secretion of macrophages was detected after stimulation/co-culturing with human apoptotic cardiocytes *in vitro* (Clancy et al 2003b), and TGFβ induced increased expression of SMAc on cultured human cardiac fibroblasts (Clancy et al 2002). Histological findings of macrophages and myofibroblasts expressing SMAc (scarring phenotype) in human fetal heart dying from CHB further supports the hypothesis (Clancy et al 2003a, Clancy et al 2004).

CHB is normally detected within 18-24 weeks of gestation, but block can be detected with ultrasonography from gestational week 16 (Lee 1990). It is not understood why fetal cardiac tissue is especially vulnerable to circulating maternal autoantibodies during this time in development, but it coincides with the time point when the AV node-His connection becomes functional (Piercecchi-Marti et al 2003).

The cardiac insult in CHB has been proposed not to be restricted to the AV node alone but to involve the entire myocardium. This is supported by the histological findings of diffuse antibody deposition, calcification and fibrosis throughout the myocardium in affected fetuses (Litsey et al 1985, Lee et al 1987, Meckler et al 1998, Piercecchi-Marti et al 2003), as well as abnormalities of the sinus atrial (SA) node (Ho et al 1986, Buyon et al 2001). Sinus bradycardia has been demonstrated in both otherwise healthy infants born to mothers with anti-Ro autoantibodies (Cimaz et al 2000) and in murine models of passive immunity with human anti-Ro antibodies (Mazel et al 1999), and after perfusion of rabbit heart with sera from mothers with CHB children (Restivo et al 2001, Fesslova et al 2003).

Findings of first-degree block detected at birth, which can progress postnatally despite the clearance of maternal antibodies from the neonatal circulation suggest that the pathogenic reaction could continue after birth (Geggel et al 1988, Saleeb et al 1999, Askanase et al 2002). Maternal antibodies might be essential during the initial phase of the inflammatory process, which then might progress into a self-sustaining process independent of circulating autoantibodies.

2.4.6 Proposed mechanisms for congenital heart block

The pathological cascade resulting in fibrosis and AV block in fetuses subject to autoantibodies directed against Ro and La has not yet been defined at a molecular level. Maternal antibodies are clearly necessary but not sufficient to develop CHB. In addition,

the Ro protein is intracellular and not accessible to maternal antibodies, and that most infants born to mothers possessing the candidate antibodies have no or mild effects on their conduction system is difficult to explain. Several mechanisms involving both direct binding to the Ro52 protein and cross-reactivity with cell surface antigens, have been suggested to link maternal autoantibodies to the inflammatory reaction and final cardiac injury characteristic of CHB.

2.4.6.1 Antibodies bind directly to Ro52

Accessibility of the Ro52 protein to the immune system might be explained by an increased protein expression during fetal development, incorrect cell migration and/or sorting, or possibly be connected with apoptotic events taking place in the fetal organogenesis and development. Apoptosis is a selective process, and in developing rat hearts has been demonstrated to affect scattered single cells rather than tracts of continuous cells (Takeda et al 1996). Although apoptosis normally does not evoke inflammatory reactions this pathway has been proposed as a potentially pathogenic process whereby Ro and La proteins may be exposed extracellularly in fetal heart tissue and become targets for circulating maternal antibodies, initiating an inflammatory response. Surface expression of Ro and La after induction of apoptosis has been reported both *in vitro* in cultured keratinocytes (Yu et al 1996) and human fetal cardiocytes (Miranda et al 1998), as well as *in vivo* where La was demonstrated to be translocated to the surface of apoptotic cells in fetal a murine heart and conduction system (Tran et al 2002). Additional observations suggest increased apoptosis, diffusely scattered, in fetal heart of fetuses dying with CHB compared to normal abortuses (Buyon et al 2003a).

2.4.6.2 Antibodies cross-react with a cell surface antigen

Another possible scenario, besides interaction with Ro52, is that the maternal autoantibodies cross-react with another protein expressed in fetal cardiac tissue. This hypothesis was supported by a report that antibodies reactive with the second extracellular loop of the serotoninergic 5-hydroxytryptamine (5-HT) receptor, cloned from human adult atrium, can bind to Ro52 (Eftekhari et al 2000). However, others have not been able to confirm the 5-HT receptor as a target of the immune response in mothers with affected children (Buyon et al 2002).

Several publications have shown arrythmogenic effects of anti-Ro52 antibodies and evidence is emerging to support a direct effect of the antibodies on cardiocyte function, possibly due to cross-reactivity. This hypothesis has been supported by the demonstration that human affinity purified anti-Ro52 positive sera induce AV block in whole young rabbit hearts (Garcia et al 1994), and human fetal hearts (Boutjdir et al 1997), and inhibit inward calcium fluxes across cell membranes (Garcia et al 1994, Boutjdir et al 1997). More specifically, maternal antibodies have been proposed to interact with the pore-forming α1-subunit of calcium channels possibly leading to internalization with subsequent cell death and exposure of intracellular Ro and La proteins, ultimately resulting in an inflammatory reaction (Xiao et al 2001b).

Whether the binding of maternal autoantibodies is direct or indirect the question remains why the fetal heart is selectively vulnerable and why CHB only affects a few fetuses in Ro seropositive women.

2.4.7 Genetic aspects

To understand the linkage between maternal antibodies and fetal cardiac injury several issues have to be taken into account. The mother can express several clinical manifestations or be totally asymptomatic. Furthermore, a mother can have a child without CHB after giving birth to an affected child despite intrauterine exposure to identical highly specific antibodies. In addition, there are several reports of mono- and dizygotic twins and triplets discordant for disease (Harley et al 1985, Lee et al 1987, Watson et al 1994, Buyon et al 1998, Eronen et al 2000, Fesslova et al 2003, Solomon et al 2003). Clearly, transferred anti-Ro/La autoantibodies cannot alone explain the pathogenesis of CHB. The genetic constitution of the child has been proposed as a potential additional factor.

Susceptibility to most autoimmune diseases is affected by a variety of genetic and environmental factors. Many autoimmune disease have been linked to a specific HLA pattern. The haplotype B8/DR3, is linked to pSS and high titers of antibodies against Ro and La, have been found to be typical for mothers with CHB children (Lee et al 1983, Watson et al 1984, Arnaiz-Villena et al 1989, Buyon et al 1990, Brucato et al 1995, Julkunen et al 1995, Kassan 1998). However, there is no significant HLA-association distinguishing between CHB and non-CHB outcome in Ro/La positive mothers or their children to date (Miyagawa et al 1997, Miyagawa et al 1999, Siren et al 1999a, Siren et al 1999b).

Non-HLA genes have also been proposed to play a role in predisposition of CHB, and an association between cytokine polymorphism and CHB has been proposed after demonstrating a significantly increased frequency of polymorphism in TNFα and TGFβ comparing 40 CHB children with healthy controls (Clancy et al 2003a). Notably, histological investigations of heart tissues from the affected fetuses demonstrated no expression of TNFα protein. TGFβ protein expression was demonstrated both intra- and extracellularly in septal regions of fetuses dying with CHB. A TGFβ polymorphism was detected in codon 10 (leucine → proline) in CHB children, accompanied by expression of TGFβ in the fetal heart. However, conclusions are difficult to draw from these experiments since the actual polymorphism evident in CHB patients have previously been associated with lower TGFβ synthesis *in vitro* and *in vivo* (Awad et al 1998).

Findings of maternal cells in heart tissues of 4/4 investigated patients with CHB children might suggest that specific maternal microchimerism can occur in neonates (Stevens et al 2003). The detected maternal cells in heart sections expressed sarcomeric α-actin, a specific marker for cardiac myocytes, indicating a cellular plasticity of the maternal cells entering the fetal circulation. The direct implication of the observed microchimerism is not clear considering that this phenomenon was additionally detected in heart sections of two out of four controls. How these cells might contribute to the pathogenesis or repair of cardiac tissue remains to be elucidated; they might be the actual target of the inflammatory response proceeding the fibrosis associated with complete AV block, or possibly actively contribute to tissue repair in the fetal cardiac tissue.

2.4.8 Treatment

As yet there are no common guidelines how to treat an *in utero* discovered AV block. However, there are reports of incomplete AV block discovered early during pregnancy, prohibited from progressing to a more severe stage by anti-inflammatory treatment *in utero*, as discussed below. A third-degree block seems to be permanent however (Saleeb et al 1999, Shinohara et al 1999).

Current treatments include *in utero* administration of steroids, plasmapheresis, sympathomimetics, and cardiac pacing. Administration of fluorinated steroids that cross the placenta in an active form, are thought to inhibit the inflammatory reaction in the fetal heart tissue. Prophylactic treatment with fluorinated steroids of women at risk has not been favored since today's predictability is low and adverse fetal and maternal effects of the treatment are feared (Baud et al 1999, Costedoat-Chalumeau et al 2003). Prenatal use of glucocorticoids starting before gestational week 16 has been suggested to reduce the risk for CHB in the fetus (Shinohara et al 1999), although there are reports of CHB despite steroid treatment (Buyon et al 1995). Reversal of incomplete block has been reported through administrating a relative high dose fluorinated steroids (Carreira et al 1993, Buyon et al 1995, Rosenthal et al 1998, Saleeb et al 1999, Theander et al 2001), although double-blinded, placebo-controlled studies are still lacking.

Plasmapheresis is a method aimed toward removing the maternal antibody repertoire and thereby decreasing exposure of maternal antibodies and reducing inflammation in fetal heart tissue (Buyon et al 1988, van der Leij et al 1994). Data concerning treatment of discovered CHB are limited by the small number of treated patients and the absence of proper control groups, so extended future trials are needed.

In utero administration of sympathomimetics has been demonstrated to increase the fetal ventricular rate, but has a poor outcome and has not been shown to revert the block (Schmidt et al 1991, Yoshida et al 2001).

CHB is a potentially life-threatening disease and has a substantial associated morbidity. Pacemaker implant is applied in cases of complete AV block, both *in utero* and in neonates. Pacing is associated with great risks and is generally not performed unless severely required. Studies to predict *in utero* death in CHB or the need for post-natal pacing has been undertaken, although no reliable predictive factors were identified (Groves et al 1996). However, reports of high risk of sudden cardiac failure at any age with only poor prognostic signs support pacing of adult patients with CHB (Michaelsson et al 1995).

3 OBJECTIVES OF THE PRESENT STUDY

Autoantibodies are important markers in clinical diagnostics and are used as tools for isolating, characterizing and elucidating the function of intracellular autoantigens. A present challenge is to explore the mechanisms whereby autoimmune diseases are initiated and to elucidate the role of autoantibodies in the pathogenic process. This thesis work focuses on the autoimmune diseases Sjögren's syndrome (SS) and congenital heart block (CHB), and the associated production as well as pathogenicity of autoantibodies directed against the Ro52 protein.

Production of anti-Ro52 antibodies in Sjögren's syndrome

The salivary glands of patients with SS are subject to chronic inflammation and these are suggested to be a site for ectopic lymphoid neogenesis. Another characteristic of SS is positive Ro or La serology, and such autoantibodies have been suggested to have an active role in the chronic inflammatory disease process. In order to understand the role of the target organ in this autoimmune disease and to relate the organ involvement to the production of autoantibodies, several studies of salivary glands were undertaken, the specific aims being:

- To investigate the structural and functional properties of the lymphocytic infiltrates in order to define their cellular composition and resemblance with secondary lymphoid organs

- To study localization and frequency of autoantibody producing plasma cells to define the role of the target organ in propagation of the disease process

The role of anti-Ro52 antibodies in congenital heart block

CHB is a serious and often lethal disease and is assumed to arise from transplacental passage of maternal autoantibodies against Ro and La initiating an inflammatory response in the fetal heart tissue. Little is known of the development of CHB, and no specific maternal antibody profile or target molecule(s) in the fetal cardiac conduction system has been identified. To date there are no general guidelines of how to treat an *in utero* discovered AV block, and clinical trials as well as more knowledge of the disease course are needed to evaluate therapies available and to define new specific strategies. In order to explore the role of Ro/La autoantibodies and to further define the mechanisms underlying this passively acquired disease several studies have been undertaken addressing clinical as well as molecular aspects, the specific aims being:

- To define the natural course of CHB

- To identify a specific serologic marker associated with development of CHB

- To define the mechanism for CHB development at the cellular level

- To develop an animal model of CHB

4 RESULTS AND DISCUSSION

4.1 ANTI-Ro52 ANTIBODIES IN SJÖGREN'S SYNDROME, PAPERS I-III

One of the classification criteria for SS is the presence of lymphocytic infiltrates in the salivary glands. The mononuclear infiltrates, which mainly consist of $CD4^+$ T cells while 5-15 % are B cells, develop in periductual and acinar areas and may become extensive, replacing large portions of the glands. The mechanism underlying recruitment of lymphocytic infiltration leading to chronic inflammation of the salivary glands in patients with SS is not clear. Positive serology for Ro and La autoantibodies is another characteristic of SS, although a role of the antibodies in disease pathogenesis has not been defined. However, demonstration of autoantibody-producing plasma cells in the salivary glands might imply a pathogenic role for these autoantibodies in the inflammatory disease process. Within this thesis work interest has been focused on the cellular structure and functionality in relation to the infiltrates in salivary glands, as well as to define the local autoantibody production to these features (papers I-III).

4.1.1 Germinal center-like structures in salivary glands

Germinal centers (GCs) normally arise in primary follicles in secondary lymphoid organs, and are critical in the development of normal B cell immune responses, providing an infrastructure for driving B cell proliferation, maturation, selection and final differentiation into plasma cells and B cell memory cells. However, ectopic GC formation has been observed in non-lymphoid organs in a variety of human diseases characterized by chronic inflammation, including RA, Hashimoto's thyroiditis and myasthenia gravis (reviewed in Hjelmström 2001), extending the biological function of GC reactions to a pathogenic role in the autoimmune response.

SS is characterized by chronic inflammation of the salivary glands. The glandular lymphocytic infiltration is thought to be a progressive feature (Jonsson et al 1993), and if extensive the infiltrating cells may replace large portions of the organ. Structures in salivary glands of SS patients resembling organized lymphoid tissue, including GC-like structures, have been reported in several studies (papers I-II and Aziz et al 1997, Stott et al 1998, Xanthou et al 1999, Prochorec-Sobieszek et al 2004). These findings suggest lymphoid neogenesis occurs in the salivary gland and a possibility that the target organ actively participates in propagating the inflammatory disease. In papers I-III we have investigated structural and functional properties of the lymphocytic infiltrates in salivary glands. Paper II includes the first study investigating the frequency of GC-like structures in salivary glands of SS patients, and these highly specialized structures were detected in 17% (28/165) of the patients, exemplified in Figure 5.

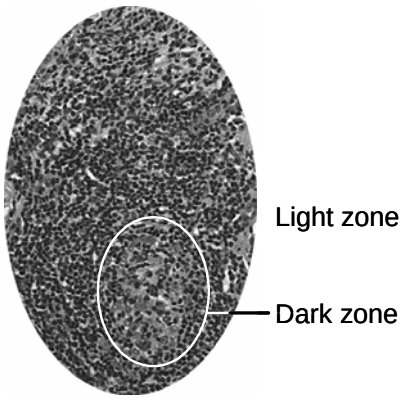

Figure 5. GC-like structure in a large inflammatory infiltrate of a salivary gland from a SS patient, demonstrating an aggregate of proliferating cells (the "dark zone").

To further investigate cellular and functional similarities with secondary lymphoid organs the morphological studies were complemented by carefully chosen molecular studies. Besides cell composition, cell proliferation, apoptosis, presence of autoantibody-

producing cells, expression of homing chemokines and adhesion molecules were investigated in SS glands using immunohistochemical methods, Table 2.

Table 2 Cellular markers used exploring structural and functional properties in GC-like structures in salivary glands of patients with SS in paper II

Cell type/event studied	Cell surface marker
T cell	CD3
B cell	CD20
Proliferating B cell	Ki-67
FDC	CD35
Activated endothelial cell	CD31
Autoantibody-production	biotinylated Ro52, Ro60 and La protein
Chemokine-expression	CXCL13, CXCL12, CCL21
Adhesionmolecules	ICAM-1, VCAM, VLA-4, LFA-1
Apoptosis	studied by TUNEL

In patients with GC-like structures the infiltrating T and B cells were found to be compartmentalized into separate areas, and further exploration of the microenvironment revealed aggregates with proliferating cells associated with a network of FDCs and activated endothelial cells. In contrast, patients lacking GC-like infiltrates in the salivary glands only demonstrated a diffuse scattered pattern of FDCs. Observations of a network of FDCs in the central part of heavily inflamed glands in patients with SS conform with previous studies, however, these FDCs have been suggested to be phenotypically different from FDCs expressed in tonsils (Aziz et al 1997).

Autoantibody-production, found to co-localize with apoptotic cells, and expression of chemokines and adhesion molecules will be discussed in detail and related to other reports in the following sections.

4.1.1.1 Local production of autoantibodies in the target organ

A positive serology for the Ro and La autoantibodies is closely associated with SS. The role of autoantibodies in the disease course is not established. However, there are reports of a correlation between antibody levels and disease activity (Wahren et al 1998, Hassan et al 2002), although there is a report not confirming this (Praprotnik et al 1999). Furthermore, local antibody production in the salivary glands has been reported both indirectly by detection of IgA autoantibodies in saliva (Horsfall et al 1989) and by extraction of autoantibody-producing cells from salivary gland tissue (Halse et al 1999), as well as directly by immunohistochemistry using biotinylated antigen in salivary gland sections (papers I-III and Tengnér et al 1998).

We investigated autoantibody-production in relation to GC-like structures to further elucidate the resemblance with secondary lymphoid tissue in regard to functionality, i.e. to production of disease-associated autoantibodies in the target organ. Autoantibody-producing plasma cells were located at the margin of large infiltrates and in interstitial spaces (papers I-III). Furthermore, glands with GC-like structures demonstrated significantly higher production of anti-Ro and La autoantibodies, and Ro/La antibody production was associated with higher levels of autoantibodies detected in sera (papers II and III). However, it should be emphasized that plasma cells producing Ro and La autoantibodies are likely to reside in other tissues as well, although we have focused on

the local production in salivary glands and detectable levels in sera throughout this thesis work

Apoptosis is a constitutive process in lymphoid tissue, used as a discard pathway for lymphocytic cells failing during selection and maturation. We detected only moderate levels of apoptotic cells in salivary glands, although somewhat more frequently appearing in patients with GC-like structures (paper II). Interestingly, apoptosis was encountered in the margins of large infiltrates and in interstitial spaces, partly co-localizing with autoantibody-producing cells. Low numbers of apoptotic cells in the glands of SS patients have been previously reported (Ohlsson et al 2001), and a recent report of decreased apoptosis in BAFF-expressing B cells (Szodoray et al 2003) supports the suggestion that B cells might escape apoptosis and proper selection in the proposed ectopic GC.

In addition, both antigen-driven clonal proliferation of B cells and clonally expanded lymphocytes and plasma cells can be identified in the salivary glands (Stott et al 1998), further supporting a hypothesis of ectopic lymphoid neogenesis in the target organ of SS patients.

4.1.1.2 Chemokines expressed in salivary glands

Chemokines are considered as key players in coordinating lymphocyte traffic throughout the body. They are constitutively expressed within lymphoid organs, directing movements during lymphocyte development and differentiation. A number of chemokines and chemokine receptors are critical for development and maintenance of lymphoid tissue. CCL21, a T cell homing chemokine expressed on HEVs, has a central role in T cell migration into lymphoid tissue, as supported by findings that CCL21 deficient mice demonstrate defective T cell recruitment into lymph nodes (reviewed in Cyster 1999). Migration of B cells into lymphoid tissue and normal polarization of B in follicles is dependent on expression of CXCL13 in the follicles as well as expression of its receptor CXCR5 on circulating B cells. Experimental studies show that CXCR5 deficient mice lack normal lymphoid organs. CXCL12 was first acknowledged as a pro-retention chemokine and later to have a central role in directing the movements of antibody secreting B cells (reviewed in Cyster 2003).

To further explore similarities with functional GCs in secondary lymphoid tissue, expression of the homeostatic chemokines CXCL13 and its receptor CXCR5, CCL21 and CXCL12 were investigated in the salivary glands of SS patients (papers I and II). The B cell-homing chemokine CXCL13 was primarily observed in acinar and ductual epithelial cells (papers I and II), while its receptor CXCR5 was expressed on infiltrating mononuclear cells in all salivary glands from patients with SS, but in none of the healthy control subjects (paper I). CXCL13 expression on epithelial cells has been reported by others (Xanthou et al 2001), but contradicted by results from Amft and colleagues demonstrating CXCL13 expression in endothelial cells in five SS patients (Amft et al 2001). Expression of CCL21 and CXCL12 was also evident in epithelial cells in all SS patients (paper II), although expression of CXCL12 was not only restricted to patients, in agreement with an earlier report (Amft et al 2001). Notably, no correlation was determined between chemokine expression and presence of GC-like structures. Restriction of CXCL12 and CCL21 expression to SS patients might suggest a constant chemokine expression level during the entire inflammatory process in the glands, possibly mediating a sustained lymphocyte infiltration of the glands. In conformity with others our data suggest epithelial cells might actively participate in the inflammatory process in the glands of SS patients by producing chemokines (Cuello et al 1998, Amft et al 2001, Xanthou et al 2001), cytokines (Fox et al 1994) and expression of MHC II (Jonsson et al 1987). In conclusion, expression of homeostatic chemokines in salivary glands of SS

patients suggests that the target organ actively participates in sustaining chronic inflammation by attracting lymphocytes.

4.1.1.3 Adhesion molecules expressed in salivary glands

Lymphocyte migration into lymphoid or inflamed tissues is not random but is rather targeted by specific adhesive interactions between lymphocytes and endothelial cells. This extravasation is a critical regulatory point in the immune system, and is a multistep process involving selectin-supported rolling of lymphocyte to endothelial cells, followed by a triggering event and firm integrin-mediated adhesion before transendothelial migration occurs. We investigated the expression of the integrins VLA-4 and LFA-1 expressed on lymphocytes, which interact with adhesion proteins from the Ig-family, VCAM-1 and ICAM-1 respectively, expressed on endothelial cells as well as APCs (paper II). These adhesion molecules are critical for the transendothelial migration of lymphocytes and have been proposed to be important for lymphocyte migration from blood to inflamed glands from studies in NOD mice (Mikulowska-Mennis et al 2001). All adhesion molecules were highly expressed in lymphocytic infiltrates in the salivary glands, suggesting activation and an active recruitment of lymphocytic cells, in concordance with previous reports (St Clair et al 1992, Saito et al 1993, Kapsogeorgou et al 2001). Glands with large GC-like structures demonstrated lower expression of LFA-1 and its ligand ICAM-1 on mononuclear cells in the central part of the infiltrates, indicating decrease in LFA-1-mediated adhesion interactions as infiltrates are being organized. Furthermore, the ratios of ICAM-1 and VCAM-1 expressing cells in relation to the total number of focal mononuclear cells were increased in glands with GC-like structures, indicating a higher degree of activation and lymphocyte trafficking in these glands.

4.1.2 Ectopic lymphoid neogenesis in salivary glands?

Secondary lymphoid organs are highly organized structures providing an environment in which antigen-specific B cells can proliferate rapidly forming a GC, mature and differentiate into plasma cells. These tissues are constantly circulated by lymphocytes in a complex process regulated by expression of an array of cytokines, chemokines and adhesion molecules. Morphological as well as several functional features characterizing a secondary lymphoid organ have been observed in salivary glands from SS patients, presented within this thesis work and by others. Recognized phenomena in the inflamed glands involve presence of germinal centers with distinct B and T cell compartments, expression of chemokines and adhesion molecules mediating homing of naïve cells, antigen-driven clonal B cell proliferation and local production of autoantibodies. In conclusion, observations indicate that salivary glands in SS patients contain elements required for perpetuating the autoimmune response. Ectopic lymphoid neogenesis in the salivary glands where ordinary regulatory mechanisms do not function might be crucial for maintaining the chronic inflammatory process in the target organ of SS patients, including local autoantibody production, and might eventually result in destruction of the organ.

4.2 ANTI-Ro52 ANTIBODIES IN CONGENITAL HEART BLOCK, PAPERS IV-X

Maternal autoantibodies against the Ro and La proteins are actively transported into the fetal circulation, which might lead to clinical manifestations including potentially life-threatening CHB. A main issue in understanding the underlying mechanisms for CHB is to extrapolate the initial antibody insult with the final cardiac injury, taking into account the facts that the target antigens are intracellular and that most infants born to mothers with these autoantibodies lack clinical signs of AV block.

CHB is a relatively rare disorder encountered by clinical immunologists, cardiologists, rheumatologists and obstetricians. During the time period of this thesis work we have established a number of both national and international collaborations with specialists within these areas in order to collect sera and clinical records from Ro/La positive mothers and children with and without clinical signs of AV block. Our work has been directed to gain wider knowledge of the pathogenic mechanisms underlying the course of CHB, addressing molecular as well as clinical aspects in order to develop better screening, risk-assessment and specific therapies for the disease.

4.2.1 *In utero* treatment of incomplete blocks

The AV block is thought to result from an inflammatory reaction in the fetal heart tissue initiated by maternal Ro/La antibodies. This reaction might progress and lead to fibrosis and calcification of cardiac tissue, resulting in an irreversible third-degree AV block. We have reported two cases of fetal AV block II, which have been successfully treated with fluorinated glucocorticoids (papers IV and VII). Our observations confirm previous reports of early treatment of incomplete block with fluorinated steroids preventing progression of or reverting the block (Carreira et al 1993, Buyon et al 1995, Rosenthal et al 1998, Saleeb et al 1999).

The first case of AV block II was discovered in gestational week 19, and the mother was treated with dexamethasone throughout the pregnancy (paper IV). The block was reverted from II to I and remained so throughout the pregnancy. The first-degree block was confirmed by ECG after birth. In a second case an AV block II was discovered in gestational week 23 and bethametasone was administrated throughout the pregnancy (papers VII and X). The block reverted to an AV block I *in utero* and remained stable during the fetal period. To our surprise the first-degree block was spontaneously reverted after birth, which has not been previously reported. Spontaneous reversement, as demonstrated in paper VII, and advancement of block after birth (Geggel et al 1988, Saleeb et al 1999, Askanase et al 2002) imply that the healing process as well as the inflammatory reaction in the fetal cardiac tissue might proceed after birth and that circulating maternal antibodies are only crucial in the initial phase of the block.

In utero treatment of an AV block is directed towards decreasing the inflammatory response in the fetal tissue and thereby prevents the block from proceeding into fibrosis and permanent scarring. *In utero* administration of fluorinated steroids is preferred since it is thought not to be metabolized by the placenta in any larger extent and thereby will be available to the fetus in an active form (Blanford et al 1977). Others have suggested that non-fluorinated steroids are comparable in this matter (Levitz et al 1978), and studies on how well compounds are transferred to the fetus during the first trimester are needed.

CHB is a rare condition, and most mothers with Ro and La autoantibodies deliver children with no detectable signs of AV block, which is why preventional treatment is questionable considering the potential adverse effects for both fetus and mother. In our study one-third of the Ro/La positive fetuses demonstrated conduction abnormalities, although most were transient affections reversible without treatment (this will be further discussed in the section below). However, we have retrospectively examined records of

Ro/La positive women treated with low doses of prednisolone (3-15 mg per day) during and/or 6 months before pregnancy. Interestingly, none of these pregnancies was complicated by CHB (paper VI). Prospective double-blinded placebo-controlled trials are needed to draw any firm conclusions regarding efficiency of *in utero* treatment with steroids, although data collected by us and others so far suggests that fluorinated steroids should be considered for fetuses with incomplete second-degree blocks. However, administration of fluorinated steroids requires close evaluation of efficacy and fetal safety, and considering recent reports bethametasone should possibly be preferred to dexamethasone (Baud et al 2001). Serial echocardiograms are recommended for monitoring fetal progress. In agreement with others (Saleeb et al 1999, Shinohara et al 1999), we were not able to reverse an established complete AV block III (the same case included in paper VII and X), even if therapy was administrated shortly after initiation of the severe block.

4.2.2 The course of congenital heart block

Prior to our studies the natural history of block development was not established, although it was suggested to develop through stages starting as an incomplete block, which subsequently might progress to a permanent third-degree block. A gradual development of the conduction abnormality would stress the importance of early discovery to initiate treatment minimizing tissue damage caused by the inflammatory reaction. The advancement of fetal echocardiography has resulted in a method to detect AV block *in utero*, and today Doppler is the method by which conduction abnormalities and/or myocardial injury can be detected the earliest.

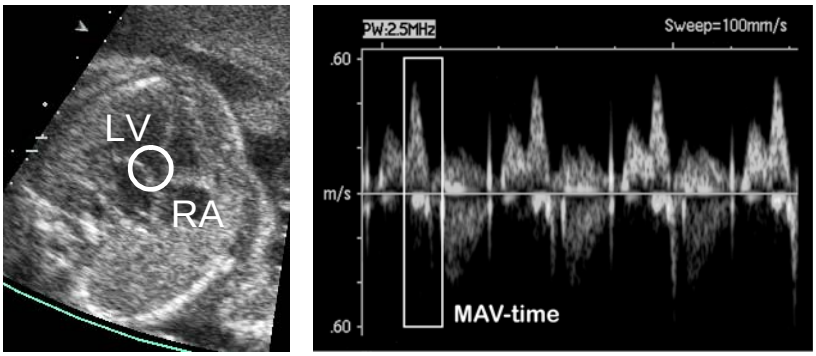

Figure 6. The fetal AV time interval was measured using two different Doppler techniques. To the left; an echocardiography representation of a fetal heart illustrating the view for mitral valve/aortic outflow measurement (LV: left ventricle, RA: right atrium). To the right; a recording where the AV time interval measuring blood flow from the intersection of the mitral wave to the beginning of the ventricular ejection wave (MAV-time) is illustrated.

To better understand the clinical course in development of CHB we prospectively followed fetal progression by measuring AV time intervals in the fetal heart using two different but equally efficient Doppler techniques (paper VII). Twenty-four Ro/La positive pregnancies were followed during susceptibility weeks 18-24. This unexpectedly revealed that fetuses with Ro52 positive mothers as a group were slightly affected when compared to the AV time intervals measured in 304 women with normal pregnancies. As many as 1/3 of the 24 fetuses with Ro/La positive mothers monitored demonstrated signs of first-degree AV block *in utero*. Our data indicate that many more fetuses subject to circulating maternal anti-Ro52 antibodies than previously known are affected, and that

antibodies might induce a general inflammation although only a proportion of the fetuses develop an AV block. However, most first-degree blocks reverted spontaneously before or shortly after birth, further indicating that there is a maturation and/or healing process proceeding through the entire fetal stage which possibly continues after birth. This might explain the spontaneous reversion of 4/4 AV block I persisting at birth, but not detected when a second ECG was taken one month later.

From both clinical and histological findings the inflammatory insult resulting in an AV block has been suggested not to be restricted to the AV node alone, but to be of a more general nature involving the sinoatrial (SA) node and the entire myocardium. Our findings of a slightly decreased heart rate in fetuses with Ro52 positive mothers might indicate involvement of the sinus node, which was supported in a passive transfer model in pregnant mice in which human anti-Ro and anti-La autoantibodies induced bradycardia and first-degree block in pups (Mazel et al 1999).

In conclusion, the pathway leading to fibrosis and cardiac conduction abnormalities is associated with anti-Ro52 antibodies but might be variable and involve not only the AV node but might also affect the entire conduction system. The inflammatory reaction seems to be kept under control in most fetuses, being subclinical in others (first-degree block) and to be full-scale resulting in fibrosis and permanent damage in a few (complete block).

4.2.3 A specific maternal antibody profile associated with block

Development of congenital heart block is associated with maternal autoantibodies against the intracellular proteins Ro and La, which can be detected in nearly all mothers with affected infants. A specific maternal antibody profile correlating with CHB would enable identification of mothers at high risk for complications with CHB, and might help to determine the pathogenic mechanism that induces this autoimmune condition. Several systematic analyses have been undertaken to identify a subpopulation or specificity of the maternal anti-Ro and La antibodies associated with development of CHB. However, the collected data is somewhat heterogeneous and the association of CHB and antibodies to the Ro52, Ro60 and La proteins vary according to the laboratory methods employed. The use of different immoassays, including ELISA (both commercial kits and in-house set-ups), immunoblot and counterimmunoelectrophoresis, displaying different sensitivities and specificities, and that patient groups tested are often small are all factors that have to be taken in account when interpreting these data.

We have investigated the antibody profile against Ro52, Ro60 and La in maternal sera associated with CHB in several studies, using western blot and ELISA with recombinant proteins and synthetic peptides (papers IV-VII and X). A universal presence of antibodies targeted towards the Ro52 autoantigen was demonstrated in all patients with CHB children. Findings of a significant association between anti-Ro52 antibodies and CHB agrees with others (Buyon et al 1993, Julkunen et al 1993, Brucato et al 1995, Dörner et al 1995, Silverman et al 1995, Julkunen et al 1998), although previous attempts to define a specific antibody profile focusing on other and larger parts of the Ro52 protein failed to demonstrated a unique anti-Ro52 antibody response (Buyon 1993, Julkunen et al 1993, Meilof et al 1993, Silverman et al 1995, Dörner et al 1996). This discrepancy could be explained by conformational differences in the different sets of peptides used in the different studies. The need of essential aa for correct structural folding is illustrated in our studies on the highly overlapping peptides p197 (aa 197-232) and p200 (aa 200-239), where antibodies in the majority of the patient sera tested recognize p200 while p197 has no or very poor recognition. Conformational studies demonstrate that p200 has a higher α-helical contribution and is more stable compared to p197 (paper VIII).

In concordance with many others we demonstrated a major antigenic region present in the central part of Ro52 (Blange et al 1994, Buyon et al 1994a, McCauliffe et al 1994, Kato et al 1995, Dörner et al 1996). Epitope mapping using overlapping synthetic peptides covering the immunodominant region, Figure 7, revealed an association between antibodies against aa 200-239, p200, and development of CHB (paper V). Additionally, antibodies against the adjacent peptide covering aa 176-196, p176, was associated with less risk of CHB. Supporting these data, we found both the p200 antibody levels and a high p200/p176 antibody ratio to be significantly associated with prolonged AV time intervals in a prospective study monitoring maternal antibody profile and fetal progress in 25 pregnant Ro52 positive women (paper X).

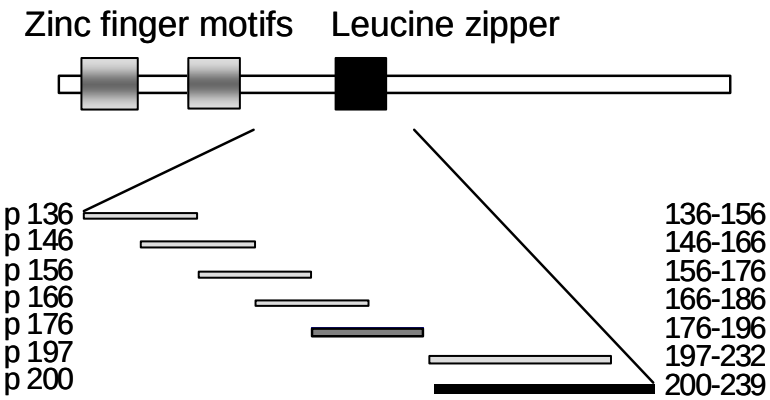

Figure 7. A schematic drawing of the overlapping synthetic peptides used for epitope mapping of the Ro52 protein. Maternal antibodies targeted toward p200 (aa 200-239) were associated increased risk for disease, while an antibody profile dominated by antibodies against p176 was predictive for an unaffected infant.

Furthermore, our results demonstrate that antibodies to Ro60 and La have a minor role in predicting the risk of CHB, which is in conformity with previous publications (Buyon et al 1989, Waltuck et al 1994, Silverman et al 1995, Julkunen et al 1998). Although CBH may develop independently of maternal antibodies against Ro60 and La these autoantibodies might, if present, be able to amplify the immunological response after onset in affected fetuses. No association was determined between disease and antibodies against La or antibodies of IgM or IgA isotype directed towards any of the Ro or La proteins.

The risk for CHB has been suggested to be more closely associated with maternal SS than with SLE (Brucato et al 2001, Julkunen et al 2001a, Julkunen et al 2004), which is supported by our findings, Table 3. Summarizing all mothers monitored in our published studies (papers V-VII and X), mothers with SLE are represented in higher numbers (n=34) compared to SS (n=19), although CHB was more frequent in mothers with SS. Thirty-seven % (7/19) of the pregnancies in mothers with SS resulted in utero AV block, versus 18% (6/34) in mothers with SLE. In our studies all mothers with affected infants had anti-Ro52 antibodies, and among mothers with SS and SLE having unaffected children 75% versus 81% had detectable levels of anti-Ro52 antibodies. SS is more closely associated with a positive Ro/La serology than is SLE, which could explain our findings. Another possibility is that the genetics associated with SS in the mothers, which is partly inherited by the child predisposes for disease.

From a clinical perspective the stability of the antibody profile in mothers at risk of delivering a child with CHB is essential in order to estimate the relevance of the p200/p176 antibody ratio and at what time-point this ratio should be tested. In a retrospective longitudinal study of ten Ro52 positive women we determined that the p200/p176 antibody ratio remains stable for long periods of time, even throughout pregnancy (paper VI). Identification of a serologic marker associated with block which could be investigated very early or even before pregnancy has major clinical impact in

order to increase the chances of early detection of the block at an early stage. The identified high-risk mothers should be closely followed during pregnancy, and fetal progress monitored by echocardiography.

Table 3 Summary of mothers included in paper IV-X, including diagnosis in mothers and outcome in children.

Maternal diagnosis	Fetal outcome				
	AV block III	AV block II	AV block I	Unaffected	Percentage affected
SS (n=19)	2	2	3	12	37%
SLE (n=34)*	3	-	3	28	18%
RA (n=2)	-	-	-	2	0%
UCTD (n=2)	-	-	-	2	0%
Asymptomatic (n=7)	4	-	2	1	86%

UCTD: undifferentiated connective tissue disease
AS: asymptomatic
*6 of these patients had in addition to their SLE a diagnosis of secondary SS, and one of these mothers had a baby affected with an AV block III

We monitored antibody levels using ELISA with recombinant Ro and La proteins and synthetic Ro52 peptides. A methodological limitation of our study is that binding of proteins and peptides to the plastic plates in ELISA could cause the antigens to fold in a non-native manner, revealing epitopes normally hidden within the native protein. We may therefore have underestimated the presence of antibodies recognizing conformational epitopes within both the Ro and La proteins, and the immunodominant part of Ro52 mapped with 20-39 aa long overlapping peptides. Interestingly, we observed frequent binding to p200 (aa 200-239) among the patient sera tested but in most cases lack of interaction with the highly overlapping p197 (aa 197-232). Analysis of the secondary structure of p197 and p200 by circular dichroism demonstrated a higher α-helical contribution in p200 and this was found to be more stable compared to p197, suggesting that folding is important for antibody interaction with the epitope and that this folding is preserved under conditions applied in the ELISA assay (paper VIII). Furthermore, the length of peptide fragments synthesized for mapping the antibody specificity should be regarded. Short peptides can efficiently mimic linear epitopes while longer peptides might be needed to reveal conformational epitopes, and although we use overlapping peptides antigenic epitopes can be lost.

Taken together, our data indicate that the ratio of maternal antibodies against p200/p176 might be used as a serologic marker for identifying the risk of CHB, with high ratio implying a higher risk for disease. To further investigate our hypothesis concerning an association between antibodies specific for p200 and CHB we have set up both national and international collaborations to test sera collected by other groups to further evaluate our findings of anti-p200 antibodies as a serologic marker for CHB. Samples to be tested include sera from about 100 mothers enrolled in the American Research Registry for Neonatal Lupus (Buyon et al 1998), and about 70 Finnish sera from a population-based study to identify mothers with CHB children (Julkunen et al 2004). A collaboration has also been established with the Department of Clinical Immunology at Karolinska University Hospital to develop a p200/p176 assay for clinical use.

4.2.4 The role of α-p200 antibodies

Our data strongly support a pathogenic role for antibodies directed against aa 200-239 (p200) of the Ro52 protein in the disease course of congenital heart block. The central part of the Ro52 protein folds into a coiled-coil and p200 encompasses a predicted leucine zipper. Leucine zippers form an α-helical structure, where every seventh aa residue consists of a leucine which will appear every second turn facing in the same direction. The secondary structure of p200 was confirmed to fold in an α-helical manner, demonstrated by circular dichroism spectroscopy (paper VIII). Leucine zippers are commonly involved in protein-protein interactions (Paris et al 2003) and Ro52 has been suggested to participate in dimer-formation (Wang et al 2001).

Association with autoantibodies specific for a functional domain such as the leucine zipper, is not a unique feature for CHB. However, to define the exact role of the antibodies directed against p200 in development of CHB we need to learn more about the mechanism underlying its pathogenesis. Binding of anti-p200 autoantibodies might possibly mediate inhibition or decrease the functional activity of the antigen, as is evident for autoantibodies such as antibodies against RNA polymerase I inhibiting RNA synthesis or against DNA topoisomerase I causing relaxation of super-coiled DNA (reviewed in Tan 1991). Antigenic epitopes are commonly highly conserved in evolution. Comparison of the human and mouse Ro52 sequences in this region, aligned by the leucine zipper motif, reveals that aa 200-239 (p200) in the human homologue corresponds to aa 204-243 in the mouse protein and all four leucines in the leucine zipper motif are identical. Furthermore, the total aa identity is 70%, while the positive similarity is 95%.

4.2.5 Pathogenicity of α-p200 antibodies in a rat model

To verify the pathogenicity of antibodies directed towards Ro52 and the aa 200-239 peptide of Ro52 we established a rat model for CHB. Female DA rats, a strain commonly used for autoimmune models, were immunized with Ro52 recombinant protein and synthetic p200 peptide, as well as with irrelevant control protein/peptide. Antibody levels were monitored and after demonstrating a plateau in antibody levels the rats were mated and conduction abnormalities in the pups monitored by ECG on the day of delivery (Figures 8 and 9).

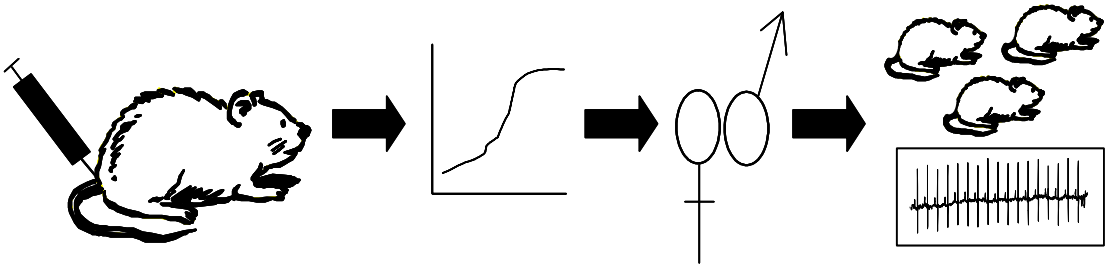

Figure 8. Model for CHB in rat. Female rats were immunized with recombinant Ro52 protein and synthetic p200 peptide, respectively. Antibody levels were monitored and after reaching a plateau the rats were mated and an ECG was performed in the pups within the first 12 hours after birth.

Figure 9. (to the right) A three-led ECG was recorded from conscious pups using silver microelectrodes attached to a body clip. Photo: Linn Horvath.

Interestingly, AV block I was detected in 19% of the pups from rats immunized with p200, supporting a pathogenic importance of anti-p200 antibodies in development of AV block. In addition, AV block was detected in 45% of the pups subject to the maternal anti-Ro52 antibodies, Table 4. Induction of AV block has previously been demonstrated in mice through both challenge with recombinant Ro52 protein (Boutjdir et al 1997, Miranda-Carus et al 1998) and by passive transfer of human affinity purified IgG autoantibodies into pregnant mice (Mazel et al 1999). Conduction effects has also been induced in rabbits after immunization recombinant proteins (Xiao et al 2001b), and after perfusing whole heart from rats as well as rabbits with affinity-purified Ro52 positive sera (Garcia et al 1994, Boutjdir et al 1998, Restivo et al 2001).

Table 4 PR intervals in pups from immunized DA rats, measured by ECG.

Rats immunized with Ro52, in total 47 pups			
Litter	PR average (ms)	PR in pups individually (ms)	No of pups with PR>PR ±2SD in ctrls*
1	60.9±2.4	65/60/61/60/59/58/63	1/7
2	65.4±3.5	66/64/65/69/58/69/67/65	7/8
3	59.8±1.9	59/57/62/59/62/60	0/6
4	66.8±7.6	73/65/63/79/65/52/72/65/67	7/9
5	65.8±5.0	65/66/58/71/69	4/5
6	59.3±6.9	67/63/53/54	1/4
7	56.6±4.3	66/58/54/54/54/59/55/53	1/8
			Σ 21/47 (45%)

Rats immunized with p200, in total 53 pups (control peptide: JB4)			
Litter	PR average (ms)	PR in pups individually (ms)	No of pups with PR>PR ±2SD in ctrls’
1	54.5±3.6	60/49/52/53/57/53/56/57	0/8
2	56.0±4.5	55/61/48/55/54/60/59	0/7
3	64.0±4.1	66/65/69/61/68/61/58	3/7
4	56.2±8.0	66/63/52/47/53	1/5
5	65.1±4.5	72/69/63/60/65/67	3/7
6	61.8±1.3	62/62/63/60	0/4
7	63.3±4.7	55/65/61/66/61/71/62/65	2/8
8	59.6±3.9	64/58/66/58/58/58/55	1/8
			Σ 10/53 (19%)

* PR(protein controls, 17 pups)=53.3±5.3
‘ PR(peptide controls, 25 pups)=58.9±3.2

Conduction abnormalities detected in both p200 and Ro52 challenged animals were restricted to AV block I, no second or third-degree blocks developing in the pups. This could be due to a too low anti-p200 antibody level in p200-immunized rats, considering that peptides often display poor antigenicity while the Ro52 protein possibly was not capable of inducing enough specific anti-p200 antibodies. Another possible explanation for the lack of severe block in our model might be that induction of antibodies against human p200 and Ro52 generates antibodies that despite having *in vitro* reactivity

(analysed in ELISA testing sera from both mothers and pups), they might not be capable of inhibiting the functional activity *in vivo*. An additional explanation could be that the DA strain does not have the optimal genetic prerequisites for CHB, related to the human relatively low reoccurrence rate in a mother with a previously affected infant (10-20%). Furthermore, lack of complete AV block has been reported in mice, and rabbit models for CHB as well (Mazel et al 1999, Xiao et al 2001b). In our murine model full-length Ro52 protein was more efficient in inducing block compared to p200, this might be related to that conformational epitopes are better conserved in the native protein resulting in induction of a stronger and more specific antibody response in Ro52 immunized animals. Another possibility is that antibodies recognizing other immunogenic parts of Ro52 and contribute to an amplified immune reaction in the fetal heart tissue.

4.2.6 Isolating human monoclonal antibodies against p200

A present challenge is to explore if anti-p200 antibodies interact directly with Ro52 or if they might cross-react with other protein(s) expressed in fetal heart tissue. For more detailed studies on localization and function of the target protein we cloned human variable domain antibody fragments using combinatorial phage libraries which were constructed using B cells from autoimmune patients (Zampieri et al 2003). We identified two clones, S3A8 and M4H1, specific for the p200 peptide (paper VIII). Epitopes recognized by these antibodies within the p200 peptide were investigated using structurally derived mutated peptides, and antibody-antigen interaction was analysed using ELISA, circular dichroism and MALDI-TOF mass spectroscopy (papers IX and X). The selected monoclonal antibody fragments recognize different epitopes within p200. M4H1 seems to recognize a linear epitope with aa 233 as the most N-terminally vital aa, while S3A8 seems to recognize a conformational epitope in the central part of p200 (Figure 10).

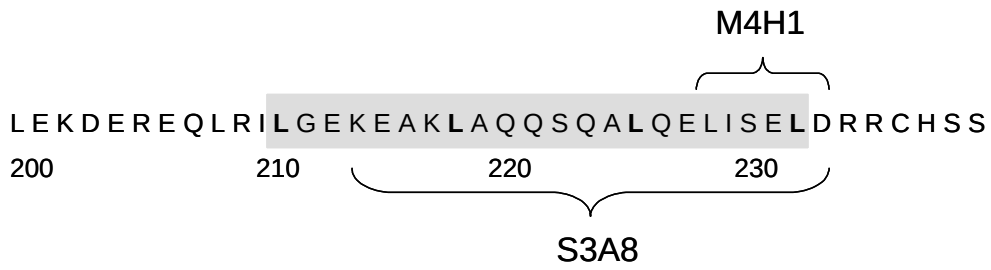

Figure 10. The p200 peptide, encompassing aa 200-239, of the Ro52 protein. The predicted leucine zipper is illustrated as a grey box (aa 211-232). M4H1 is suggested to interact with a linear epitope within aa 227-233, while S3A8 might recognize a conformational epitope within aa 214-233 (paper IX).

Furthermore, the major part of the anti-p200 activity within sera from mothers and children with a history of CHB was generated by antibodies with a S3A8-like specificity, and only a minor part by M4H1-like antibodies (papers IX and X). Generation of monoclonal anti-p200 antibodies became valuable tools for further detailed studies of the mechanism behind CHB, described in the section below.

4.2.7 α-p200 antibodies disturb calcium homeostasis

The association between anti-p200 antibodies and fetal conduction abnormalities both in humans and in our rat model supports a pathogenic role for anti-p200 antibodies in the course of CHB. However, their exact role in the course of disease and a pathway that might result in organ failure in a proportion of developing fetuses remains to be defined. Several publications reporting arrythmogenic effects of anti-Ro antibodies in animal models have appeared in the literature. IgG-enriched fractions from human sera containing anti-Ro antibodies were used in a superfusion assay in newborn rabbit ventricular papillary muscles, and specifically altered the action potential consistent with an alteration in calcium flux (Alexander et al 1992). This treatment also reduced the inward current in isolated rabbit ventricular myocytes using the patch-clamp technique (Garcia et al 1994). Furthermore, CHB has been induced in human fetal heart by perfusing with affinity purified Ro52 sera of mothers with CHB children, resulting in inhibition of L-type calcium currents in fetal cardiac cells (Boutjdir et al 1997).

To address whether anti-p200 antibodies could have a direct effect on cardiomyocytes with regard to calcium homeostasis primary cultures of neonatal rat cardiomyocytes were incubated with anti-p200 specific antibodies and intracellular calcium levels were monitored. These studies revealed that application of S3A8 antibodies led to severe dysregulation of calcium homeostasis and cell death by apoptosis. Similar results were obtained when applying sera from p200-immunized rats to the cell cultures, while M4H1 antibodies did not affect the calcium levels in the cultured cardiomyocytes. Taken together, our experimental findings suggest that anti-p200 antibodies interact with cardiomyocytes and disturb calcium homeostasis. These findings support and extend previous suggestions of an interaction between cell surface protein regulating intracellular calcium transport and pathogenic antibodies operating in CHB.

4.2.8 Do α-p200 antibodies cross react with a cell surface antigen?

We and others have demonstrated a link between anti-Ro52 antibodies and disturbed calcium transport in cardiomyocytes. However, a pathway including accessibility of the intracellular Ro52 antigen to extracellular maternal autoantibodies resulting in disturbed calcium homeostasis in fetal cardiomyocytes does not seem likely. An alternative to direct involvement is that anti-Ro52 antibodies might cross-react with cell-surface protein(s) expressed in the target tissue. A candidate for this cross-reactivity is the L-type calcium channel, suggested by Boutjdir and colleagues after demonstrating inhibition of L-type calcium channels *in vitro* in fetal cardiomyocytes using affinity purified IgG from mothers with CHB children (Boutjdir et al 1997), and a reduced L-type calcium channel density was reported in pups of Ro52 immunized mothers in the rabbit (Xiao et al 2001a) and mice (Qu et al 2001). The long-lasting (L)-type calcium channel is a voltage-gated ion channel supporting inward current of calcium, and plays a role in triggering cardiac and smooth muscle contraction. These channels are ubiquitously expressed in the cardiovascular system, and are crucial in action potential propagation and conduction in the sinus and AV node in the heart. The L-type calcium channel is a complex of five different polypeptides, α1, α2, β, γ and δ. An interaction between maternal autoantibodies and the pore-forming subunit α_{IC} of L-type calcium channels has been suggested, a cross-reactivity supported by interactions between positive IgG and the α_{IC}-subunit detected by immunoblotting (Xiao et al 2001a). Notably, neither of the other two isoforms of α_I, or other subunits of the large calcium channel complex were tested for interactions in the actual study. Although several animal models and experiments using fetal cardiomyocytes demonstrate that anti-Ro52 antibodies affect the function of the L-

type channels there is no convincing *in vitro* data supporting a physical interaction between antibodies and L-type calcium channels. Furthermore, no mono-specific antibody has been shown to cross-react with the L-type calcium channel, affinity purified IgG fractions from CHB mothers having been used as a source of anti-Ro52 antibodies in all experiments. Another question to be addressed is the association with a certain time window of susceptibility for fetal block, between gestational weeks 16-24 and how this might be correlated with expression of L-type calcium channels in the fetal cardiac tissue. However, L-type calcium channel density is lower in fetal rabbit heart cells than in adult cardiac cells (Huynh et al 1992), implying that a blockade of calcium channels by maternal autoantibodies might impose a great burden for the fetal heart and explaining why the adult heart rarely is affected by anti-Ro antibodies.

Our experimental data do not support a mechanism involving a direct interaction with the calcium ion channel complex as suggested by Boutjdir and colleagues, preliminary data (R Klauninger). The overload in calcium seen in cardiomyocytes treated with anti-p200 monoclonal antibodies, as well as sera from p200-immunized rats, implies another mechanism than that stated above. Our results suggest that anti-p200 antibodies, with the fine-specificity profile of S3A8, constitute the pathogenic antibodies in CHB inducing increased levels of intracellular calcium. However, this may also explain findings by Boutjdir and colleagues of an inhibitory effect of Ro52 positive sera on L-type calcium channels, as increased intracellular levels of calcium inhibit these ion channels (Findlay 2004). We propose a pathway where anti-p200 antibodies bind cardiomyocytes directly altering the calcium homeostasis resulting in cell death. Further work has to be undertaken to identify the interaction partner(s) for anti-p200 antibodies, and to define the pathway by which the overload in calcium is generated.

4.2.9 Proposal of a mechanism for congenital heart block development

The mechanisms underlying CHB are just emerging, and several cellular and molecular pathways have been proposed to explain the complex pathogenesis of the disease and how it is associated with maternal anti-Ro52 antibodies. Based on our findings we propose a two-step mechanism in which maternal anti-p200 antibodies with S3A8-like specificity are assigned a pathogenic role by binding to cardiomyocytes and inducing the apoptotic process by disturbing the calcium homeostasis (illustrated in Figure 11). An interaction is initially established between circulating maternal anti-p200 antibodies and a thus far unknown protein expressed on the fetal cardiomyocyte cell surface. This interaction mediates disturbance in calcium homeostasis resulting in intracellular calcium deposition and subsequent cell death. The vulnerability of the fetal heart tissue within the “time window” reaching from gestational week 18-24 might be associated with specific developmental stages in the fetal cardiac tissue, and has to be addressed in experiments investigating properties of fetal cardiomyocytes during this time. A possibility is that although maternal antibodies enter the fetal circulation at an early stage there might be a threshold level needed for induction of insult, and that this time point coincide with certain differential stages of the cardiac cells affected in a case of block development. It is not likely that anti-p200 antibodies are selectively transported into the fetal circulation but coexist with antibodies specific for other parts of Ro52 as well. Findings of a p200/p176 antibody ratio might suggest that in mothers with a high ratio, that is high p200 and low p176 titers, the anti-Ro52 response is scewed towards anti-p200 recognition and that the ratio is an amplification of this effect. This predisposition for producing anti-p200 antibodies might in turn be genetically linked. Another possibility is that the anti-p176 antibodies is that they have a protective effect,

possibly by competing with anti-p200 for binding the target molecule without mediating any functional effect.

In a second phase, there might be one or several fetal factors distinguishing affected from the unaffected fetuses. In most cases the apoptotic events induced bring about a mild inflammatory reaction that is controlled by most fetuses. The inflammatory reaction might be visible as a first-degree block, which spontaneously reverts or passes by unnoticed if not associated with bradycardia and is therefore not investigated. However, in a few cases the inflammation is not controlled and might become chronic. This might involve recruitment of inflammatory mediators, extended apoptosis and possibly involve exposure of intracellular Ro and La proteins to circulating maternal autoantibodies, further sustaining the inflammation. The fetal cardiac tissue would be subject to fibrosis, calcification and scarring resulting in a permanent block. This model implies that the fetal factor involved influence the reaction conducted upon the inflammatory response, possibly linked to the fetal immunogenetic composition.

Our model is confirmed by clinical observations in human and supported by in vitro findings, performed both in our rat model for CHB and *in vitro* experiments using monoclonal antibodies specific for p200 and primary rat cardiomyocyte cultures. However, there are several issues that remain to be addressed. Above all, it will be important to identify the interaction partner expressed on fetal cardiomyocytes interacting with anti-p200 antibodies and to verify that S3A8-like antibodies can induce block *in vivo*, as well as to define the fetal factor distinguishing fetal vulnerability for inflammation initiated by maternal anti-p200 antibodies. Another interesting question at issue is the role of the specific anti-p200 antibodies in the mothers, whether they are pathogenic in her disease course as well or might be associated with certain clinical manifestations.

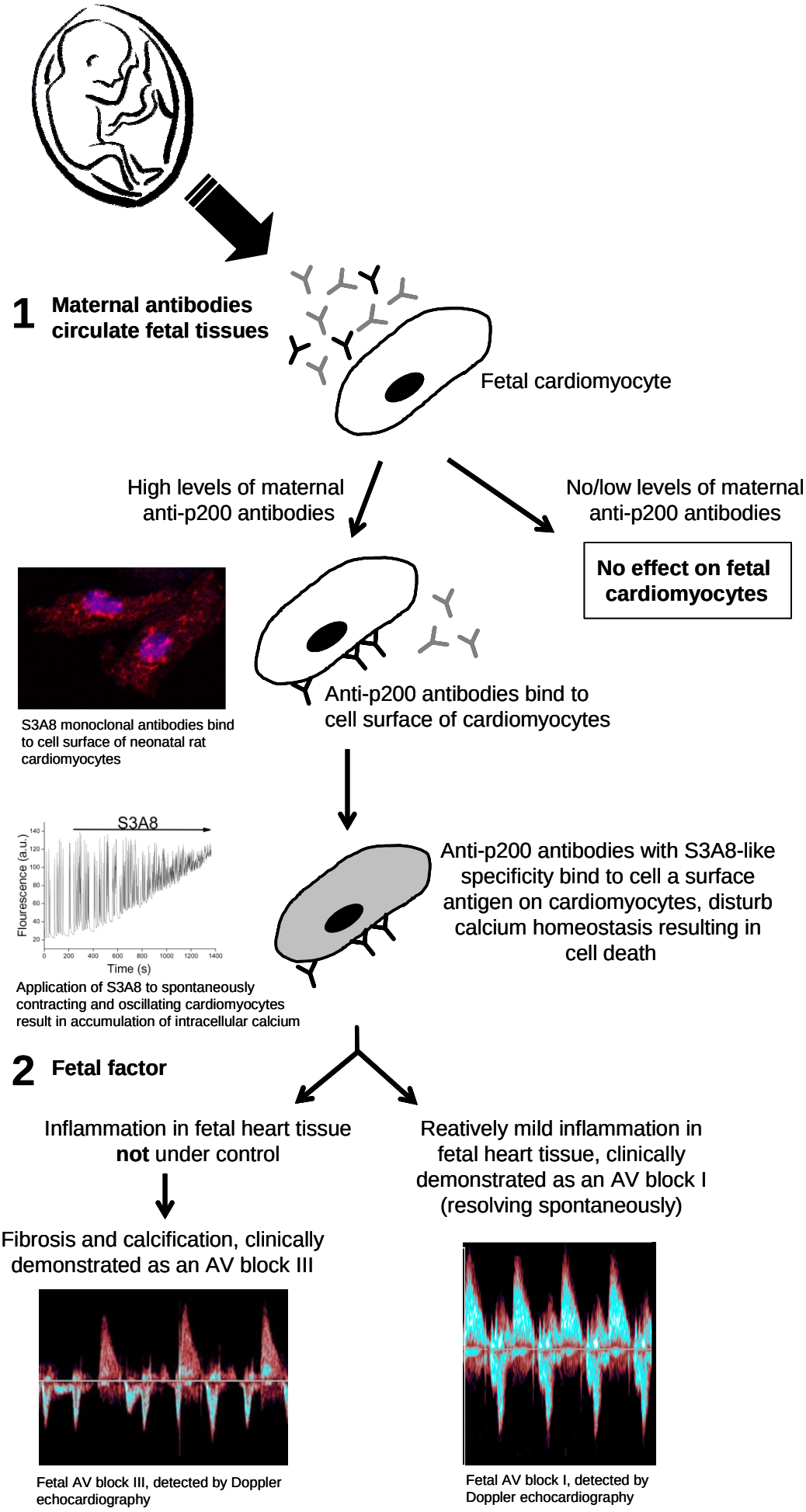

Figure 11. Proposed mechanism for development of congenital heart block (CHB).

Table 1 Diagnosis and classification criteria applied for patients in papers I-X.

Paper	No. of patients	Diagnosis	Classification criteria
I	14	pSS (n=10)	1
		sSS; SS/SLE (n=3)	1, 2
		sSS; SS/RA (n=1)	1, 2
II	165	pSS (n=91)	3
		sSS (n=50)	3, 2, 4, 5, 6
		Not established' (n=24)	
III		pSS (n=9)	3
		sSS; SS/SLE (n=3)	3, 2
		Sicca symptoms	
IV	1	pSS	7
V	43	pSS (n=11)	1
		sSS; SS/SLE (n=7)	1, 2
		SLE (n=13)	2
		Asymptomatic* (n= 4)	
		CHB (n=8)	
VI	16	pSS (n=5)	3
		SLE (n=6)	2
		APL (n=2)	8
		MCTD (n=2)	9
		UCTD (n=1)	
VII	24	SS (n=8)	3
		SLE (n=11)	2
		RA (n=2)	4
		UCTD (n=1)	
		Asymptomatic* (n=2)	
X	25	See paper VII, one additional asymptomatic* patient included	

SS: Sjögren's syndrome, SLE: systemic lupus erythematosus, RA: rheumatoid arthritis, APL: anti-phospholipid syndrome, MCTD: mixed connective tissue disease, CHB: congenital heart block, UCTD: undifferentiated connective tissue disease, CREST: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias

'Records not available/sufficient to classify patients as either pSS or sSS, all had focus score ≥ 1 (number of infiltrates in minor salivary glands with >50 mononuclear cells per 4 mm^2)

*Positive serology for Ro52 but no clinical signs of rheumatic disease

1 The preliminary European Community criteria for SS (Vitali et al 1993)

2 American College of Rheumatology criteria for SLE (Tan et al 1982)

3 Revised European criteria for SS (Vitali et al 2002)

4 The American Rheumatism Association criteria for classification of RA (Arnett et al 1988)

5 Criteria for polymyositis (Bohan et al 1975)

6 Criteria for symptoms associated with rheumatic conditions, such as CREST syndrome, primary biliary cirrhosis, chronic active hepatitis (Klieppel 1997)

7 Copenhagen criteria for SS (Manthorpe et al 1986)

8 Preliminary classification criteria for definite APL (Wilson et al 1999)

9 Criteria for MCTD according to Alarcon-Segovia and Cardiel (Alarcon-Segovia et al 1989)

5 CONCLUDING REMARKS

The objectives of this thesis were to study the production and localization of anti-Ro52 autoantibodies in salivary glands from patients with Sjögren's syndrome, as well as the pathogenic effect of these antibodies in the passively acquired condition congenital heart block.

Chronically inflamed salivary glands from Sjögren patients were investigated for morphological, structural and functional properties associated with ectopic lymphogenesis. We could demonstrate high expression of adhesion molecules and B and T cell homing chemokines in association with large lymphocytic infiltrates with germinal center-like structure, while autoantibody-producing plasma cells co-localized with apoptotic cells in the margins of these infiltrates. Findings of autoantibody production in the target organ were associated with high antibody levels in sera. Taken together our studies suggest that the salivary glands of Sjögren patients might be subject to ectopic lymphoneogenesis, including functional germinal centers and local antibody production. Our results indicate that the target organ plays an active role in propagating the chronic inflammatory response.

The potential pathogenic importance of anti-Ro52 antibodies was investigated *in vivo* in pregnant women at risk of delivering a baby with the life-threatening condition congenital heart block and also in a murine model of the disease, as well as *in vitro* in primary rat cardiomyocyte cultures. From our work both clinically applicable findings and molecular mechanistic features have emerged. For the first time it has been demonstrated that congenital heart block develops gradually, documented by monitoring fetal progress with Doppler echocardiography. This is an essential finding for therapeutic design and understanding of the mechanisms underlying disease. Thorough epitope mapping of humoral responses against the Ro and La proteins revealed a specific maternal anti-Ro52 antibody profile associated with fetal conduction abnormalities. Maternal antibodies directed against aa 200-239 (p200) of the Ro52 protein, including a leucine zipper, are predictive for disease. Identification of a stable serological marker for development of congenital heart block enables risk-assessment in the mothers, and increases the chances for early detection and successful fetal outcome. The pathogenic role of the specific anti-p200 antibodies was verified in a rat model of the disease. Using human anti-p200 monoclonal antibodies isolated by a phage display technique, we could demonstrate an arrythmogenic effect of antibodies recognizing a specific epitope within the p200 peptide on cardiomyocytes mediated by disturbing the calcium homeostasis in the cells. The demonstrated interaction might be a result from cross-reactivity with a cell surface antigen expressed on the cardiomyocyte cell surface. In conclusion, we propose a mechanism for development of congenital heart block in which specific anti-Ro52 antibodies interact with proteins expressed on cardiomyocytes and affect their function.

Even though a pathogenic role of anti-Ro52 antibodies has emerged through this thesis work, there are still many questions to be addressed, including further exploration of the clinical impact of anti-p200 antibodies as target molecules in development of congenital heart block and to identify the cell surface interaction partner on fetal cardiomyocytes.

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