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Natural and Pathologic Autoantibodies

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1. Introduction

Detection and characterization of autoantibodies reacting with self-antigens is generally used in laboratory diagnostics. However, the presence of different autoantibodies in the blood serum doesn't mean automatically a pathologic condition. Autoantibodies are present both in different diseases as autoimmune diseases, chronic inflammation or infections, and in healthy individuals without any symptoms. The present paper discusses the detailed analysis of recognition pattern and fine epitope specificity of these autoantibodies to better understand of their occurrence and evolution, and their role in physiologic and pathologic conditions.

1.1 Evolution of the immunological recognition

Microorganisms present in the environment continuously come into contact with the human body through external or internal surfaces. Most microorganisms are neutral or useful, but others – so called pathogens - are dangerous for the other living beings including human individuals. During evolution all multicellular organisms have developed defence mechanisms capable of eliminating these invading pathogens without causing damage to self structures. All vertebrates and invertebrates manage self and non-self discrimination. Consequently, discriminating self from non-self is of key importance for directing immune functions effectively, operating on the basis of distinct recognition systems. Any attempt to answer questions concerning recognition must consider the universality of receptor-mediated responses. These may be designated to two forms: pattern recognition receptors and rearranging clonally distributed antigen-specific receptors that distinguish between self and non-self.

1.1.1 Pattern recognition as a basic immune function

Innate immunity serves as first line of defence against pathogens. Its early evolutionary appearance is indicated by its presence in all multicellular organisms including plants, invertebrates and vertebrates. Since invertebrate species rely on innate defence mechanisms only, survival of the species in the presence of environmental pathogens is achieved at the level of the population, which means that individual members, up until a fraction of total population, are dispensable (Kvell et al., 2007). Innate immunity uses receptors that are ancient in their evolutionary origin. These non-clonally distributed receptors have to be able to recognize a wide variety of molecular structures associated with pathogens without

damaging self-structures. The problem lies in the discrepancy between the vast heterogeneity of pathogens and the limited number of possible recognizing receptors in the genome. This implies that the relatively few available specific receptors must recognize structures shared by large groups of pathogens, and that the recognized structures have to be pathogen-specific molecular patterns rather than particular molecules specific for pathogens. These pathogen associated molecular patterns (PAMPs) are conserved products of microbial metabolism, they are highly glycosylated, and are essential for microbial survival. The receptors recognizing these PAMPs are termed pattern recognition receptors (PRRs). We distinguish three functional classes of PRRs: endocytic receptors such as cellular C-type lectins, scavenger receptors and Mac-1 (CD11b:CD18), which facilitate opsonisation and phagocytosis. This type of recognition is predominantly based on sugar-sugar interactions. The second set of PRRs are secreted proteins including mannose binding lectin, C1q, pulmonary surfactant proteins A and D, C-reactive protein and lipopolysaccharide binding proteins, respectively. These molecules facilitate opsonisation for phagocytosis and aid the complement system in destroying pathogens that have been bound by these secreted proteins (Medzhitov 2001). The third functional group is constituted by signalling receptors such as the Toll-like receptors (TLRs), which activate several intracellular signalling cascades, eventually leading to the activation of many immune response genes. PAMPs are targets for many PRRs in innate immunity. PRRs are expressed on cells positioned strategically in the first line of pathogen encounter such as surface epithelia, marginal zone of spleen, and on antigen presenting cells (APCs) such as macrophages and dendritic cells. It is important to note that the relatively broad spectrum of ligands recognized by TLR family members also includes glycoproteins, which points toward the adaptive recognition system (Klein & Nikolaidis 2005). Thus, the TLR family possibly represents an important milestone on the way to a recognition system characteristic for adaptive immunity (Cooper et al., 2006).

Recognition of PAMPs can activate direct effector mechanisms of innate immunity such as phagocytosis, secretion of antimicrobial peptides and induction of nitric oxide synthase in macrophages. Activation of innate immunity results in the secretion of several inflammatory cytokines such as interleukin-1, interleukin-6, tumor necrosis factor- α , type I interferon and many chemokines. One of the most important events caused by PAMPs recognition is the surface expression of CD80 (B7.1) and CD86 (B7.2) co-stimulatory molecules on APCs, which is necessary for the priming of T-dependent adaptive immune responses. Therefore in addition to activate direct first line defence mechanisms, innate immunity substantially contributes to the adaptive response as well. It is important to note that while PRRs recognize molecular patterns instead of specific molecules, and significant redundancy and promiscuity exists in the molecular nature of the recognized ligands, PRRs discriminate infectious non-self from self perfectly. One plausible explanation for this is that PRRs were selected and genetically stabilized over an evolutionary time scale creating an advantage for survival, and organisms possessing self reactive PRRs were eventually eliminated. This process prevents autoimmunity in those organisms which have only the innate recognition system (Cooper et al., 2006; Kvell et al., 2007).

1.1.2 Antigen specific recognition

The adaptive immune system containing specialized organs (bone marrow, thymus, spleen, lymph nodes, highly structured lymphatic tissues associated with the wet and dry body

surfaces), that provide appropriate microenvironment for cells which are committed to antigen specific immune defence (T and B cells), appeared later during the evolution. It can be generally found in jawed vertebrates, however the earliest species with a variable antigen receptor based adaptive-like recognition system are jawless fish (lamprey, hagfish). These fish have non-immunoglobulin like clonally distributed receptors with leucine-rich repeats (similar to TLRs) generated with a gene rearrangement mechanism other than the recombination activating genes (RAG-1:RAG-2) characteristic for jawed vertebrates (Pancer et al., 2004). The appearance of adaptive immune system in jawed vertebrates gives the impression of a “sudden” change between jawless and jawed fish. This “big bang” hypothesis concerning gene duplication events, acquisition of a retrotransposon and the appearance of molecules such as major histocompatibility complex, T- and B-cell receptors (Abi Rached et al., 1999) has been challenged by showing that integration of minor changes accumulated over an extended evolutionary time lead to the appearance of adaptive immune system (Klein & Nikolaidis 2005). Taking into consideration the major immunological recognition and activation theories from Janeway’s self/non-self recognition to Polly Matzinger’s danger hypothesis and from Burnet’s clonal selection to Smith’s quantal theory recently, there is a trend to synthesize the self/non-self vs. danger models, particularly proving that receptors distinguish pathogen and danger signals simultaneously (Liu et al. 2009).

In vertebrates the adaptive immunity generates a virtually indefinite pool of recognizing molecules: the T and B cell receptors (TCR, BCR), which repertoire makes the adaptation of each individual to pathogenic challenges possible. According to the clonal selection hypothesis these receptors are clonally distributed, each of them represented by single cell clone. The benefit of the high number of available antigen receptors in adaptive immunity comes with the cost of potentially dangerous recognition of self-structures, leading to autoimmunity. Therefore carefully organized selection mechanisms exist to select the potentially useful clones, and to eliminate or inactivate the autoreactive ones. Germline genes encoding T and B cell receptors are rearranged by the site specific recombinases RAG-1, RAG-2. Once these antigen receptors appear on the cell surface, the cell carrying them has to survive two types of selection. The first of these is probing the utility of the expressed receptor by testing whether it is capable to recognize its ligand in the microenvironment. This selection step is termed positive selection, since in the case of the appropriate engagement of antigen receptor the cell survives. Although the process was described first and in more detail for T cells maturing in the thymus, it was also clearly demonstrated for B cell maturing in the bone marrow and spleen (Cancro & Kearney, 2004). The ligand that activates the antigen receptor is self-peptide-MHC complex and possibly soluble immunoglobulin for T and B cells, respectively. Positive selection operates on a thin margin, the strength of the signal generated by antigen receptor engagement must be lower than in full activation, thus it provides a partial activation signal. The second selection step eliminates clones that possess antigen receptors, which recognize self too strongly, and termed negative selection. This mechanism is based on the full activation of antigen receptor mediated signalling pathways by self antigens and eventually leads either to the deletion of the cell clone, or to long term unresponsiveness of the cell to subsequent stimuli (anergy). Alternatively, in the case of B cells, the recombination machinery could be re-activated and the other immunoglobulin gene harbouring allele could be rearranged (receptor editing), giving the cell a second chance to produce an antigen receptor not reacting with self

structures above threshold. Thus, the selection of antigen receptor bearing cells, irrespective of whether they belong to the T or B cell pool, is governed by interaction with self ligands instead of non-self ligands. The generation of the adaptive immune repertoire is therefore strongly self-referential (Janeway 2001).

2. Natural immunity

Since the innate recognition system discriminates self from non-self perfectly, the contribution of innate immunity to the activation of adaptive responses seems to be of vital importance for maintaining tolerance at the periphery. The appearance of co-stimulatory molecules on APC surface is critical for the activation of both T and B cells. In the absence of appropriate co-stimulation the activation signal remains below threshold level and the adaptive immune response will not be activated. The innate and adaptive arms of the immune system differ from each other in several important features and their cooperation is essential for the correct function of immune defence. As a connection bridging the evolutionarily oldest innate and the newly evolved adaptive systems a third compartment of immune machineries, the natural immune system has recently been described. A distinct set of lymphocytes – both T and B cells – with characteristic phenotypes and specialized functions participates in this system. These subsets of cells exhibit common phenotypic characteristics and possess both innate and adaptive features, suggesting a transitional stage in the immune system's evolution. The most important cellular components of the natural immune system according to recent knowledge are the invariant natural killer T (iNKT) cells, mucosa associated invariant T (MAIT) cells, $\gamma\delta$ T cells and B1 B cells. The functional character of antigen recognition by these cells (and the immunoglobulins produced by B1 B cells) are closer to the pattern recognition features than to the classical adaptive type immunological recognition, however, the recognizing molecules are genuine T and B cell surface receptors.

2.1 Cellular elements of natural immune system

Among unconventional T cells, only two subsets display both a TCR and selecting MHC class Ib molecules highly conserved between species, the iNKT cells and the mucosal associated invariant T (MAIT) cells. These two populations express highly restricted TCR repertoires consisting of an invariant TCR α chain. Both subsets are selected by hematopoietic cells expressing evolutionarily conserved non-polymorphic MHC class Ib molecules, CD1d for iNKT cells and MHC-related molecule 1 (MR1) for MAIT cells. CD1d-restricted iNKT cells and MR1-restricted MAIT cells constitute two subsets of unconventional T cells that are phylogenetically conserved. Therefore, they are thought to play an essential role within the immune system of mammals (Treiner et al., 2005).

MAIT cells are selected by MR1 in the thymus on a non-B non-T hematopoietic cell, and acquire a memory phenotype and expand in the lamina propria of the gastrointestinal tract and in mesenteric lymph nodes in a process dependent both upon B cells and the bacterial flora. Thus, their development follows a unique pattern at the crossroad of iNKT and $\gamma\delta$ T cells. These features suggest that MAIT cells could be involved in tolerance or immunity to infections in the gut. The function of MAIT cells is unknown, but intuitively we can argue that it is related to their localization in the gut mucosa. MAIT cells could somehow be

involved in the defense against orally acquired pathogens or in non-immune function important for gut mucosa homeostasis. MAIT cells might also control the type of the gut immune response and/or be involved in oral tolerance. Controlling the balance between tolerance and immune response in the gastrointestinal tract is highly important, and could explain the striking conservation of the MAIT cells across species. The functional relevance of MAIT cells is also underlined by the fact that they represent 1-4% of peripheral T cells in human blood (Treiner et al., 2005).

iNKT cells are selected, expand, and acquire their innate-like phenotype and functions in the thymus. They accumulate in the liver and the spleen, independently of the presence of any exogenous stimuli such as the normal bacterial flora. iNKT cells play an important role in both protective and regulatory responses. The nature of the response is determined by the initial cytokine environment: interaction with IL-10-producing cells induces regulatory T cell type iNKT cells and that with IL-12 producing cells results in Th1 type responses, while their production of IFN γ activates both innate and adaptive immune systems. Upon activation of iNKT cells tumor cells can be efficiently eliminated and they also play a role in the development of obesity (Lynch et al., 2009).

The $\gamma\delta$ TCR repertoire similarly to the repertoire of innate immune receptors could have been selected through evolution. Thymic selection does little to constrain $\gamma\delta$ T cell antigen specificities, but instead determines their effector fate. In general, it is believed that $\gamma\delta$ T cells recognize host antigens and play a role in epithelial cell maintenance. Intraepithelial lymphocytes (IEL) ontogeny can show minimal dependency upon the thymus, as they can escape the thymus at a very early stage and migrate into the gut mucosa where they achieve maturation. They may even develop directly from bone marrow derived precursors in specific intestinal lymphoid aggregates called cryptopatches. The absence of positive selection, and the lack of antigen specific priming, seems ideal for $\gamma\delta$ T cells to function in the first line of defence. When activated through the T cell receptor, antigen-experienced cells make IFN γ , whereas antigen-unexperienced $\gamma\delta$ T cells produce IL-17, a major initiator of inflammation. One of the main functions of IL-17 is to promote the expansion and maturation of neutrophils in the bone marrow. Therefore the rapid IL-17 response mounted by antigen-inexperienced $\gamma\delta$ T cells would play a critical role at the onset of an acute inflammatory response to pathogens that the host encounters for the first time, or to host antigens that are only revealed by injury. Furthermore, by acting early in the inflammatory response, $\gamma\delta$ T cells may affect the development of antigen specific $\alpha\beta$ T cell and B cell responses. Thus $\gamma\delta$ T cells may play a much larger role in the adaptive immune response than previously recognized. Since $\gamma\delta$ T cells contribute to host immune competence in several ways it is understandable why these cells have been maintained throughout vertebrate evolution, even when $\alpha\beta$ T cells and B cells are also present (Konigshofer & Chien 2006).

B1 B cells were originally distinguished from B2 cells on the basis of their expression of CD5, a glycoprotein marker previously considered to be T cell specific. CD5 is a type I transmembrane glycoprotein with three scavenger receptor cysteine rich domains and a highly conserved intracellular domain. Its role in signalling was extensively studied both in T and B cells. As it is associated with antigen receptor signalling complexes, the CD5 molecule considered to be a negative regulator of TCR and BCR signalling. Later on a CD5-B1 B cell population was also identified and termed B1b B cells. Differences in the function

and developmental requirements of the two B1 B cell subgroups are poorly characterized; however, it seems that the BCR/CD19 complex is of crucial importance in developmental decisions between B1a and B1b B cells (Haas et al., 2005).

In addition to surface phenotype, B1 B cells have several unique properties distinguishing them from conventional B2 cells. B1 B cells represent a self-renewing population found in high number in the peritoneal and pleural cavities, while they are virtually absent from peripheral lymph nodes and can be found in low number among splenic B cells. They are long lived in vitro, can be forced with phorbol esters to proliferate, and they could not be activated through BCR crosslinking. The immunoglobulin repertoire of B1 B cells is restricted in the number of immunoglobulin genes used; it is dominated by rearrangement of J-proximal V genes and has significantly fewer N insertions than the repertoire of B2 cells (Kantor et al. 1997).

There is a long-standing dispute over the developmental origin of B1 B cells in literature (Haas et al., 2005). According to the lineage model, B1 B cells are generated from fetal precursors present in the fetal liver, omentum and splachnopleura. This view is substantiated by the ability of fetal precursors to reconstitute both the B1 and B2 compartments in irradiated mice, while adult bone marrow-derived cells reconstitute B2 cells only. The induced differentiation model of B1 B cell development proposes that the B1 phenotype is a consequence of T-independent-2 like activation event, thus the specificity of BCR is the key factor which determines the B1 phenotype. The differential ability of fetal vs. adult precursors to generate B1 B cells is due to the different antigen receptor repertoire of these precursors. This argument is supported by several transgenic models in which the origin and specificity of the immunoglobulin transgene determined the B1 phenotype (Chumley et al., 2000).

Functions of B1a cells include the participation in the early phases of immune responses and most importantly the production of natural antibodies with dominantly IgM isotype, which is substantiated by the ability of B1 cells transferred adoptively into irradiated mice to restore normal IgM level. These lines of evidence and the properties of B1 B cell produced natural antibodies indicate that B1 B cells represent an intermediate stage of evolution between innate and adaptive immunity.

2.2 Natural (auto)antibodies

Natural antibodies are immunoglobulins mostly of IgM isotype, and are secreted by B1 cells without immunization with antigen. These antibodies can recognize genetically conserved sequences of pathogens and may serve in the first line of immune defence during an infection. In contrast, natural autoantibodies present in the serum of both healthy humans and patients with chronic inflammatory or systemic autoimmune diseases recognize a set of self-structures that have been conserved during evolution. Most of natural autoantibodies belong to the IgM or IgG isotype, and show polyreactivity with a broad range of affinities for the recognized epitopes (Lacroix-Desmazes et al., 1998).

Several functions have been suggested for natural autoantibodies: they may participate in the selection of immune repertoires, play a role in the acceleration of primary immune responses, and the clearance of apoptotic cells, possess anti-inflammatory effects and contribute to the maintenance of immune homeostasis (Lacroix-Desmazes et al., 1998).

Discrimination of natural antibodies from natural autoantibodies is somewhat artificial since given the limited B1 immunoglobulin gene repertoire driving natural antibody production and the numerous distinct antigens recognized it is probable that specificities with self non-self cross reactivity exist. Based on the above properties of natural antibodies, these molecules could be considered as the “innate like arm” of humoral immune system (Czömpöly et al., 2008).

3. Physiologic and pathologic autoantibodies

The phenomena that natural autoantibodies could recognize self antigens which are also targeted by antibodies in autoimmune diseases are not unprecedented. Several lines of evidence indicate that antibodies recognizing factor VIII, thyroglobulin, DNA, endothelial cell membrane components etc., are present in sera of both healthy individuals and patients with autoimmune diseases. These findings raise the question whether these detected antibodies are pathologic autoantibodies or belong to the pool of natural antibodies. It is possible that the fine epitope pattern recognized by natural antibodies and disease associated autoantibodies within the targeted antigen is different.

3.1 Characterization of fine epitope structure of the antibodies

There is a need for epitope mapping on circulating autoantibodies both in the basic and clinical immunology and in the immuno-biotechnological research and development. A mixture of different natural and pathologic autoantibodies is present in human blood samples with various antigen specificity. All the classical physico-chemical and immunochemical methods used in antibody characterization are technically difficult in the case of autoantigens.

3.1.1 Methods for determination of epitope specificity

Several techniques are available for the chemical determination of fine specificity of recognition molecules; however, a large scale analysis on serum samples from healthy individuals and patients with autoimmune and other diseases is both theoretically and technically difficult. Epitope mapping with overlapping synthetic peptides is a useful technique, but its constraints include the uncertainties linked to in silico B cell epitope prediction used for selection of antigenic regions, the partial coverage of primary sequence by synthetic peptides and the possible loss of all unpredicted or conformational epitopes. Synthetic overlapping peptides are suitable in the case of well characterized autoantigens. Limited proteolysis and the following mass spectroscopic analysis are generally used techniques in monoclonal antibody characterizations. Random peptide libraries were developed for characterization of epitope specificity on circulating autoantibodies by M13 filamentous phage system. The method was optimized on monoclonal antibodies and applied for serum samples. During our further development lambda phages were used to display fragments of previously determined antigens. Bacteriophage surface display of peptides is a recently used technique for a variety of applications. This technique resembles most the physiological antigen conformation and does not require prior epitope prediction. The technology is based on the expression of recombinant peptides or proteins fused to a phage coat protein. Its key advantage is in the physical coupling of the displayed protein to

the nucleic acid coding for it, making the repeated affinity selection and amplification possible. The most commonly used systems are based on fusion to a filamentous phage coat protein. However, the life cycle of these phages limits the size of the displayed peptide, therefore we have chosen phage lambda for the epitope mapping of naturally occurring and pathologic autoantibodies in our different studies. The library contains fragments of the antigen with random starting point and length, consequently it overcomes the theoretical and technical limitations associated with pre-designed fragments or overlapping synthetic peptides (Czömpöly et al., 2008).

3.1.2 Epitope mapping of naturally occurring antibody family specific for the mitochondrial citrate synthase protein antigen

The basic structural elements of living cells such as the cytoskeleton, metabolic organelles, transporters, molecular components of transcription and translation etc., are genetically conserved. The maintenance of immunological tolerance against these structures is a basic functional duty of immune machinery in all of the three levels. The mitochondrion is absolutely necessary for eukaryotic cell function. Genetic alterations which affect mitochondrial proteins have serious consequences, if the mutation is compatible with life at all. Because of their endosymbiotic evolutionary origin, proteins compartmentalized into mitochondria represent an interesting transition from prokaryotic foreign to essential self molecules. To date there are only a limited number of epitope mapping analyses performed on human antigens that are recognized by natural autoantibodies. In particular, little is known about the possible overlap between recognized epitopes of innate and self-reactive natural antibodies. The structural and functional conservation of mitochondrial components makes them candidate antigens for detailed analysis of evolutionary connections between the innate and adaptive immune response. No classical mitochondrion-targeted autoimmune disease – with the exception of the primary biliary cirrhosis is known, suggesting a well established tolerance both at the innate and adaptive level. The inner membrane enzymes, especially the citric acid cycle enzymes offer appropriate models for testing their immunoreactivity, because they are in continuous connection with both innate and adaptive components of the immune system during physiologic turnover of cells. The immunological recognition and the immunoreactivity with these molecules are less studied, and the possible changes in physiological autoreactivity under pathologic autoimmune conditions remain largely unclear (Czömpöly et al., 2006). To address these issues we have chosen a mitochondrial inner membrane enzyme, citrate synthase (CS) as model antigen for epitope mapping using sera of healthy individuals and patients having various systemic autoimmune disease (systemic lupus erythematosus (SLE), rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyositis, systemic sclerosis (SSc), Raynaud's syndrome and Sjögren's syndrome). The CS enzyme is not only a theoretically appropriate model – this is one of the first living protein during the evolution – but has also been studied at gene, protein structure and functional levels.

We demonstrated the presence of antibodies recognizing CS in the sera of both healthy individuals and systemic autoimmune patients. The enzyme specific antibodies with IgM isotype were more frequently present in all investigated groups than those of IgG or IgA isotypes and the incidence of autoantibodies with IgM isotype was significantly higher in autoimmune patients compared to the healthy controls. We found that the reactivity against

CS of individual sera remained permanently constant over a five year period (Fig.1.), in opposite to the anti-CS antibodies with IgG isotype which showed various titer during the investigated period on the same individuals. Our findings, that the majority of these antibodies have IgM isotype, are already present in infants, and the long term stability of their serum titers in adults indicate that these specificities belong to the natural autoantibody repertoire established early in postnatal life. The occurrence of anti-CS antibodies with IgG isotype we can explain as the physiologic dynamics of normal immune defence against different pathogens.

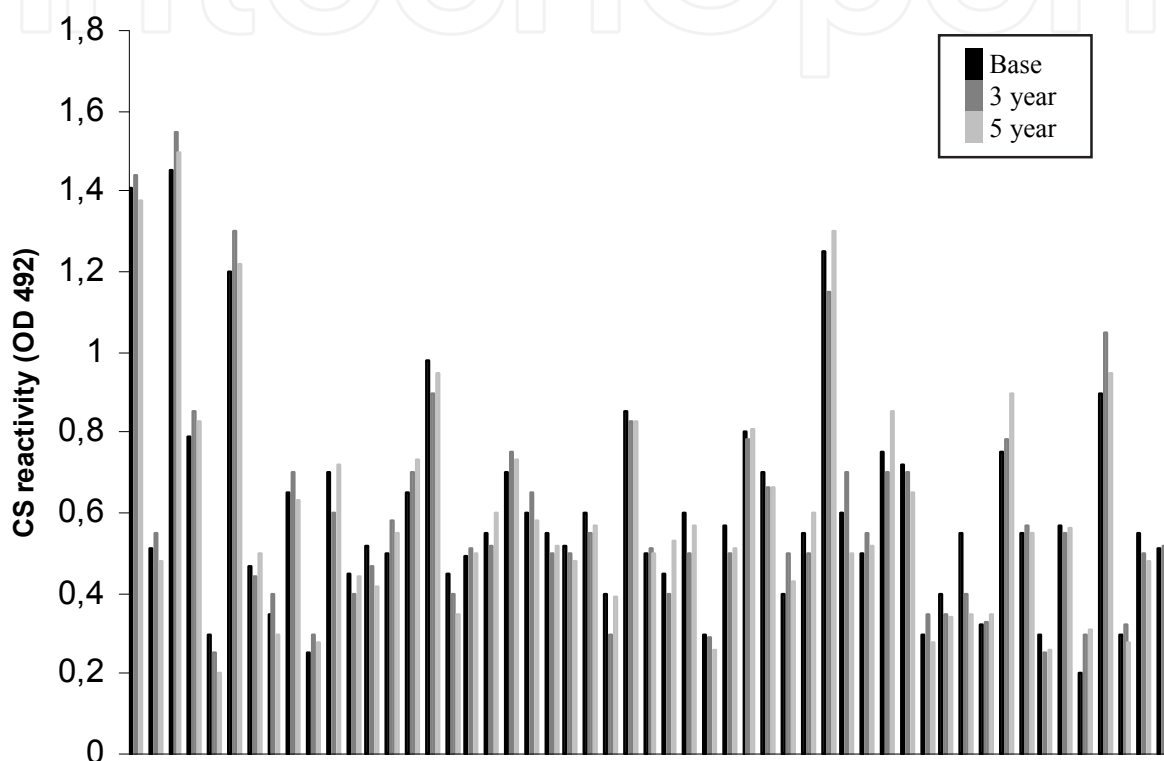


Fig. 1. CS reactivity in healthy individuals – 52 blood donors - followed up during a five year period with IgM isotype specific indirect ELISA.

To compare epitopes recognized by natural autoantibodies in healthy individuals with those recognized in systemic autoimmune patients we performed the epitope mapping of anti-CS antibodies under physiological and pathologic (systemic autoimmune) conditions. Epitope mapping with overlapping synthetic peptides is a widely used technique, but its constraints include the uncertainties linked to in silico B-cell epitope prediction used for selection of antigenic regions, the partial coverage of primary sequence by synthetic peptides and the possible loss of all unpredicted or conformational epitopes. Since these effects could have influenced our results, we sought to perform the epitope mapping using a basically different technique. Bacteriophage surface display of peptides is an extensively used technique for a variety of applications. The most commonly used systems are based on fusion to a filamentous phage coat protein. However, the life cycle of these phages limits the size of the displayed peptide, therefore we have chosen phage lambda for construction of a CS antigen

fragment library to analyze the fine epitope structure of anti-CS autoantibodies (Czömpöly et al., 2006). The library contains fragments of CS with random starting point and length; consequently it overcomes the theoretical and technical limitations associated with the overlapping synthetic peptide approach. With this phage display based approach we compared the epitope patterns recognized by anti-CS autoantibodies found in sera of healthy individuals and patients with systemic autoimmune diseases. According to our results there is no favoured region of the CS molecule recognized exclusively either by healthy individuals or patients with systemic autoimmune diseases, but the fine epitope pattern is different in the two groups examined (Fig. 2.).

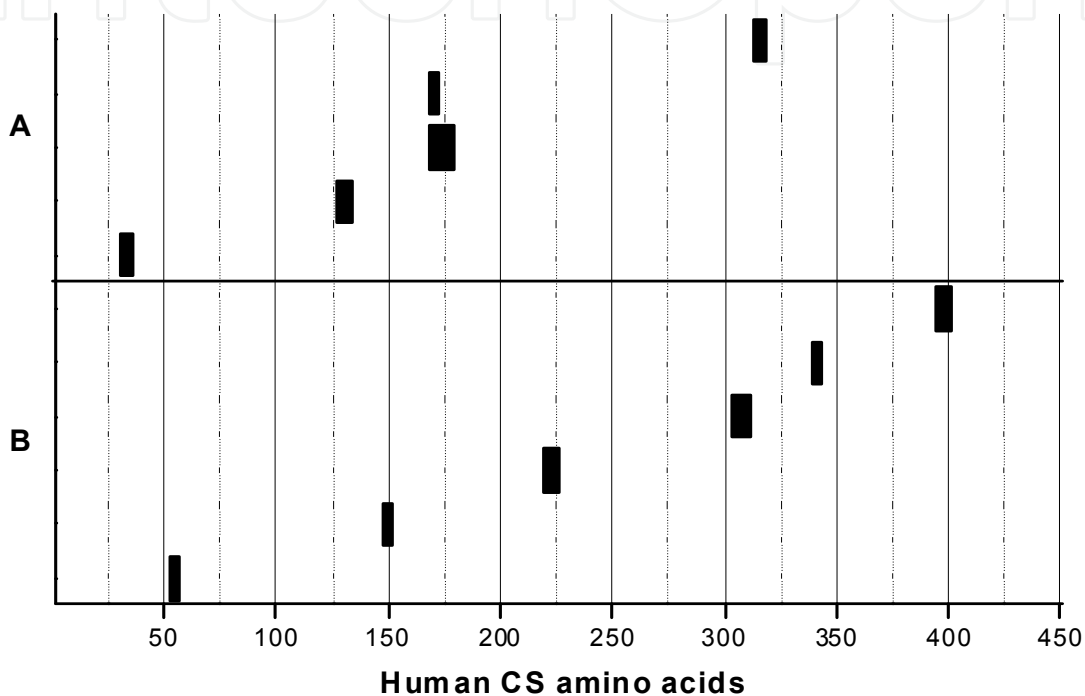


Fig. 2. Amino acid sequences of CS recognized by healthy individuals (A) and patients with systemic autoimmune diseases (B)

3.1.3 Epitope mapping of pathologic autoantibodies specific for a well conserved nuclear antigen, DNA topoisomerase I

Previously described experiments underlined the necessity for the epitope mapping of an autoantibody which is specific for a well defined pathologic condition and has a high diagnostic value. Our aim was to decide whether the target antigen of the disease associated autoantibody is also recognized by naturally occurring autoantibodies. We assumed that comparison of the epitope patterns recognized by natural and disease-associated autoantibodies would contribute to the better understanding of the differences between natural and pathologic autoantibodies. To address these issues we chose DNA topoisomerase I as a model antigen, since anti-topoisomerase I antibodies are important in the diagnosis of SSc.

Using the phage display technique previously developed in our department and optimized by analyzing epitopes of anti-CS antibodies, we performed the epitope mapping of anti-

topoisomerase I autoantibodies and examined whether the target antigen of the disease associated autoantibody is also recognized by naturally occurring autoantibodies (Simon et al., 2009).

With the help of the bacteriophage lambda library containing fragments of topoisomerase I with random starting point and length we compared the epitope patterns recognized by anti-topoisomerase I autoantibodies found in sera of patients with diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc), and SLE. The results showed that the pattern of recognized epitopes is different between dcSSc, lcSSc and SLE patients (Fig. 3).

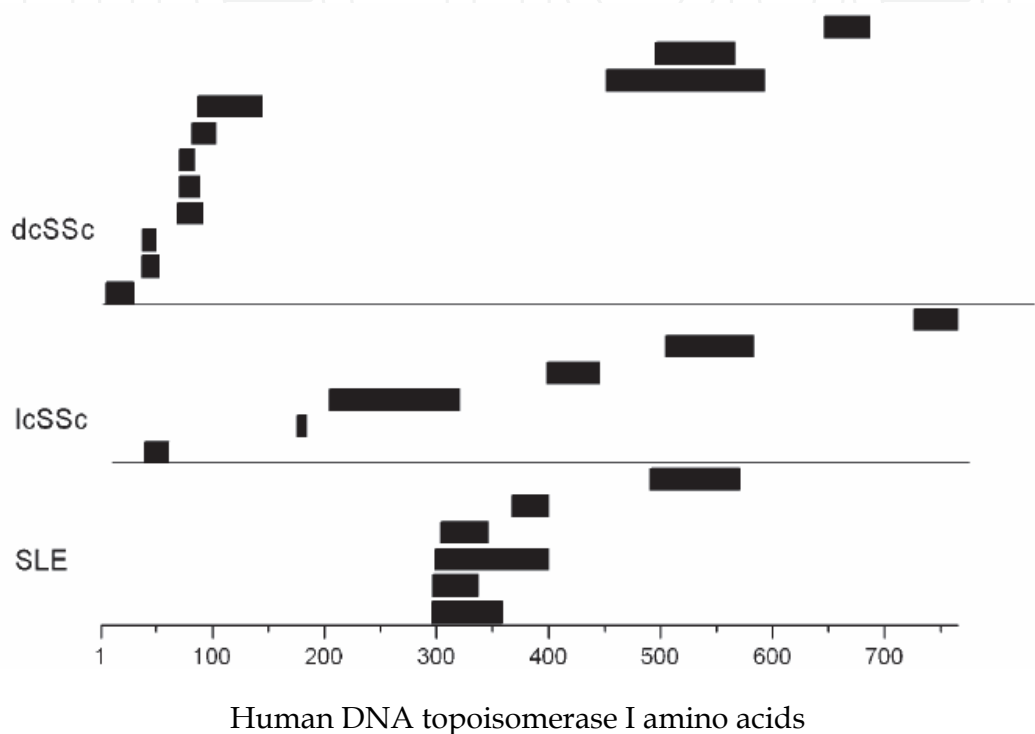


Fig. 3. The pattern of recognized topoisomerase I epitopes is different between dcSSc, lcSSc and SLE patients

A common fragment recognized by all patients' sera was located in the region of amino acid sequence (AA) 450-600, which is in agreement with previously published results. In addition to this, sera of dcSSc patients recognized several short fragments at the N-terminal part of the molecule. Previous studies performed with fusion proteins covering the N-terminal domain starting from AA 70 reported that this part of the molecule is recognized by anti-topoisomerase I antibodies. However, the opposite has also been reported by using a fusion protein covering the entire length of the N-terminal domain and showing that this part of the molecule is not targeted by anti-topoisomerase I antibodies. These seemingly contradictory results may be explained by the different methods and antigen constructs used, and most importantly by possible conformational factors, which could influence the accessibility of short epitopes buried in the tertiary structure. It is important to note that the majority of new epitope containing fragments we identified at the N-terminal part spans only 20-30 AA. The 5-25 AA fragment of the N-terminal part of the molecule contains an experimentally proven granzyme B cleavage site, thus it is possible that in vivo cleavage of topoisomerase I by granzyme B released during T cell mediated cytotoxic responses results

in the formation of a neo-antigenic determinant represented by this fragment. In vitro assays using the full length antigen or the full length N-terminal domain may fail to detect antibodies recognizing these short epitopes suggesting strong conformational sensitivity.

On the basis of fragments identified by library selection nine maltose binding protein-topoisomerase I fusion proteins were constructed and expressed (Fig. 4.).

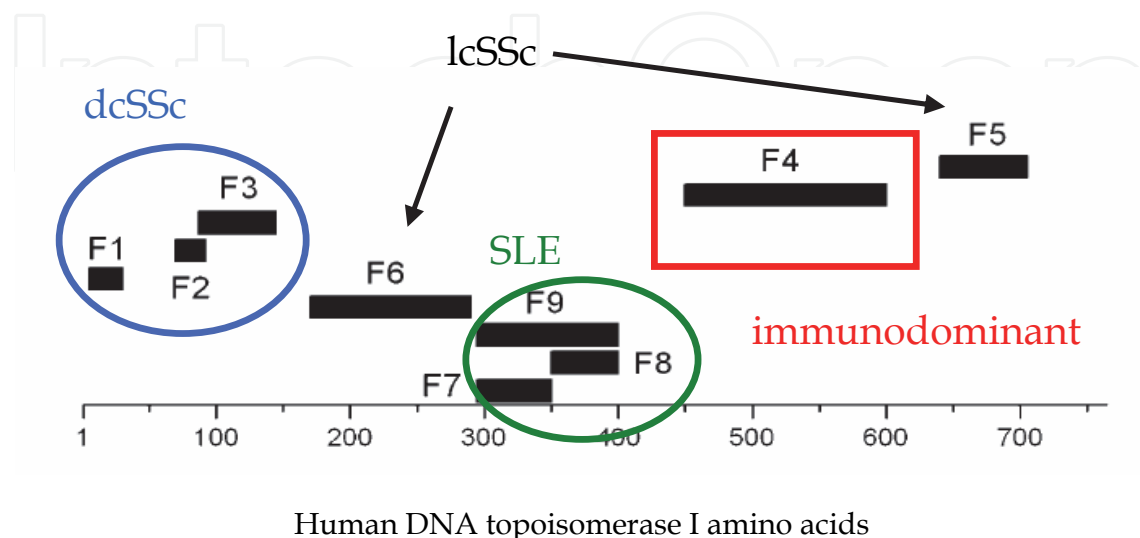


Fig. 4. The constructed and expressed maltose binding protein-topoisomerase I fragment fusion proteins

First we tested recognition of these fusion proteins with sera of healthy individuals and found that a significant portion of healthy individuals possess antibodies with IgM and IgG isotype against fragment F4. Fragment F4 represents a 150 AA long, genetically conserved sequence of topoisomerase I. Using a large number of sera we showed that the presence of antibodies against fragment F4 is essentially independent of the age and geographical origin of healthy individuals. In addition, antibodies against fragment F4 could also be detected in sera of patients with inflammatory rheumatic diseases other than SSc and SLE. Anti-F4 antibodies with IgM isotype are present in the highest titer in sera of anti-topoisomerase I antibody positive SSc or SLE patients. It is important to note that all 67 sera from anti-topoisomerase I antibody positive SSc or SLE patients were found to be positive for anti-F4 antibodies with IgG isotype, and the titer of these antibodies was the highest in this group among all groups tested. The fact that these sera were shown to be negative for anti-topoisomerase I antibody by a conventional ELISA test using the full length antigen could indicate that the sequence represented by fragment F4 could be hidden in the three-dimensional structure of the full length molecule. These findings raise the possibility that antibodies against fragment F4 present in sera of healthy individuals and patients with systemic autoimmune diseases could belong to the pool of naturally occurring antibodies. To our knowledge, these are the first results demonstrating that natural antibodies against topoisomerase I are present in human sera.

It is not unprecedented that natural autoantibodies recognize self antigens which are also targeted by antibodies in autoimmune diseases. Since anti-topoisomerase I antibodies can also be detected in sera of patients with glomerulonephritis, chronic graft versus host

disease, primer biliary cirrhosis and in some cases of chronic hepatitis C virus infection induced liver diseases the question arises whether these detected antibodies are pathologic autoantibodies or belong to the pool of natural antibodies. Fragment F4 represents a 150 amino acids long sequence of topoisomerase I, consequently it is possible that the fine epitope pattern recognized by natural antibodies and disease associated autoantibodies within this part of topoisomerase I is different.

The recognition of the majority of fragments (F2, F3, F5-7, F9) seemed to be characteristic for the individual patient sera used for library screening, instead of being characteristic for the given disease subgroup. This is in agreement with result of Henry et al., who found both individual and longitudinal differences in the recognized topoisomerase I epitopes. However, antibodies recognizing the common F4 fragment were detected in all patients' sera tested. In addition to this immunodominant part of topoisomerase I, we identified two new regions (F1 and F8) which were previously not shown to be targeted by anti-topoisomerase I antibodies. Fragment F1 (an evolutionarily relatively new sequence, specific for vertebrates) was recognized by 26% of dcSSc patients and antibodies against fragment F8 (a highly conserved sequence) could be detected in 50% of SLE patients, indicating that these fragments could represent characteristic epitopes for dcSSc and SLE, respectively. Longitudinal analysis showed that reactivity to fragment F4 was stable, while the reactivity to F1 and F8 fragments varied over time.

3.2 Comparative analysis of fine epitope pattern of natural and pathologic autoantibodies

It is possible that the presence of natural antibodies is essential for the appearance of disease associated autoantibodies, since natural autoantibodies can, under appropriate conditions; provide the templates for the emergence of higher affinity and class-switched pathogenic autoantibodies. IgG isotyped disease associated autoantibodies may recognize genetically determined epitopes (epitope patterns) and can be detected in genetically predisposed individuals, which was also suggested by a study examining monozygotic twins suffering from SLE (Silverman et al., 2008). Thus tolerance against conservative antigens might mostly be genetically determined. The permanent impairment of the development and maintenance of tolerance can lead to autoimmune disorders. Pattern recognition mechanisms were thought to be specific for innate immunity and considered to be the defence mechanisms of evolutionarily ancient species. According to our results natural autoantibodies, in terms of their antigen recognizing characteristics, resemble the pattern recognition receptors and recognize epitope patterns. Pathologic autoantibodies detected in autoimmune diseases however are directed mainly against a well defined, disease associated sequence (epitope).

4. Conclusion

A large number of circulating antibodies directed against functional structures of the cell (nucleic acid, nuclear molecules, receptors, or other functional cell components) can be detected in systemic autoimmune diseases. Their presence plays a central role in the diagnosis and classification of this kind of disorder. Moreover, several longitudinal cohort studies have shown that patients may carry autoantibodies many years before they manifest clinical symptoms and detecting these antibodies in serum has been shown to have strong

predictive value. Primary structure homologies between the antigens targeted in some autoimmune diseases and conserved sequences of different pathogens (viruses and bacteria) are well known. Although this so called “molecular mimicry” has been extensively studied, direct causality of infections in the development of autoimmune diseases has only been verified in a few patients. Apart from the homologies in primary structure, the similarities in the physico-chemical molecular shape between the mammalian antigens and the structures of microorganisms could provide a real structural basis for the biological recognition suggesting a pivotal role of three-dimensional shape of conserved antigens in both targeting type immunity and tolerance (Czömpöly et al., 2008).

According to the orthodox view of phylogenetic development, immunity has reached its zenith with the emergence of the adaptive immune system. Consequently, we tend to be influenced by anthropocentric views and overlook how other highly developed organisms manage living in hostile environments. As recently more data have become available regarding non-traditional animal models, it has been suggested that the emergence of adaptive immunity is perhaps not the culmination of the evolution of immunity, but simply a successful alternative to using innate immunity alone. For millions of years, many species could keep-up in the continuous arms-race between pathogen and host called co-evolution without the surveillance of adaptive immunity. The complexity of biology should never be underestimated as it turns out that those animals lacking RAG-dependent adaptive immunity can make up for an equal amount of diversity using highly variable elements of innate immunity finally exhibiting adaptive features. On the other hand, in vertebrates, adaptive immunity often simply serves as a sophisticated targeting device that recognizes and then processes the antigen but finally leaves the messy job of actually clearing up pathogens to the immense capacity of innate immunity. Therefore, once again we see that borders are blurring and the strict distinction between innate and adaptive immunity might need revision (Kvell et al. 2007). Network of natural immunity – including wide range of different cellular elements and naturally occurring antibodies – could explain as the missing evolutionary chain between the “classic” innate and adaptive immune system.

4.1 Clinical relevance of detection of autoantibodies

Analyzing the recognition of epitopes of natural and pathologic autoantibodies could contribute to diagnosis and better understanding of pathomechanisms of systemic autoimmune diseases. The onset of the disease may correlate with a switch from production of IgM to IgG isotypic antibodies. Nevertheless, the exact role of autoreactive IgM in the autoantibody response and the switch to other isotypes is not known. It has to be mentioned that IgM isotypic natural autoantibodies can have a role in protection from autoimmunity by facilitating the removal of apoptotic cells and increasing the tolerance of B cells to self antigen. Since one of the essential functions of the immune system is the prevention of self antigens to stimulate an inflammatory reaction, the presence of autoantibodies is the consequence of a breakdown or failure of B cell tolerance toward the corresponding autoantigens. The timing of exposure, the level of affinity of the autoreactive IgM autoantibodies and their local concentration may determine which scenario applies, i.e., autoimmunity or tolerance. Detection of autoantibodies reacting with self antigens is generally used in laboratory diagnostics. However, their presence in serum samples doesn't mean automatically a pathologic condition. Natural autoantibodies could recognize self

antigens which are also targeted by antibodies in autoimmune diseases. The immune response could be explained by a general recognition of the immunodominant part of the molecule, followed by appearance of antibodies directed against disease associated sequences (Czömpöly et al., 2009). Detection of autoantibodies recognizing different epitopes of these antigens could be a useful tool in laboratory diagnostics (Simon et al., 2009).

4.1.1 Diagnostic issues of natural and pathologic autoantibodies

Early diagnosis and initiation of adequate therapy as soon as possible is crucial in systemic autoimmune diseases such as SSc and SLE, because after insidious onset of the disease the development of internal organ manifestations can lead to death of the patient in a few years. Anti-topoisomerase I autoantibodies are considered to be associated with dcSSc. However, the presence of anti-topoisomerase I autoantibodies is not entirely restricted to this subset, since anti-topoisomerase I antibodies have been demonstrated in lcSSc, SLE and other inflammatory diseases. The fact that anti-F4 antibodies were detected in sera which were tested negative for anti-topoisomerase I antibody by a conventional ELISA kit using the full length antigen indicates that an ELISA test using recombinant F4 fragment might be a more sensitive way to determine anti-topoisomerase I positivity and could contribute to early diagnosis and monitoring the activity of SSc (Simon et al., 2009).

4.1.2 Prognostic value of epitope pattern

In systemic autoimmune diseases the prognosis is mostly determined by the activity of the disease and the extent of the developed irreversible lesions. Since anti-topoisomerase I autoantibody is found to be associated with increased mortality, pulmonary fibrosis, musculoskeletal and cardiac involvement, proteinuria and the level of anti-topoisomerase I autoantibody correlates with the extent of fibrosis of the skin and internal organ involvement in dcSSc, it may serve as an activity marker of disease (Minier et al., 2010).

Statistical analysis of clinical data (extent of skin involvement, hand contractures, azotemia and/or malignant hypertension, cardiac involvement, pulmonary artery hypertension, dysmotility and stricture/dilatation of esophagus, extent of lung fibrosis, forced vital capacity) failed to demonstrate associations between anti-topoisomerase I antibody epitope specificity and clinical presentation of the disease. This is in agreement with results also reporting lack of clear association between changes in the anti-topoisomerase I antibody response and clinical parameters (Henry et al., 2005). However, there was a significant difference between F1 negative and F1 positive groups of dcSSc patients in average age and the duration of the disease. The difference in the duration of disease between anti-F1 antibody positive and negative dcSSc patients, together with findings of our longitudinal analysis, may indicate that the anti-topoisomerase I immune response could be explained by a general recognition of the immunodominant part on the molecule (fragment F4), and the disease associated autoantibodies may target the N-terminal part later during the course of the disease. Thus autoantibodies against fragment F1 may represent a new marker of late stage dcSSc (Simon et al., 2009).

Comparison of clinical data of anti-F8 positive and anti-F8 negative SLE patients suggested that SLE patients with antibody against fragment F8 have Raynaud's phenomenon and a

milder presentation of the disease (lack of arthritis, central nervous system and kidney involvement).

Discrimination between naturally occurring and pathologic autoantibodies is available according to their recognition patterns, and this is not only a theoretical question but holds important practical – diagnostic and prognostic – consequences in the daily laboratory routine.

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6. References

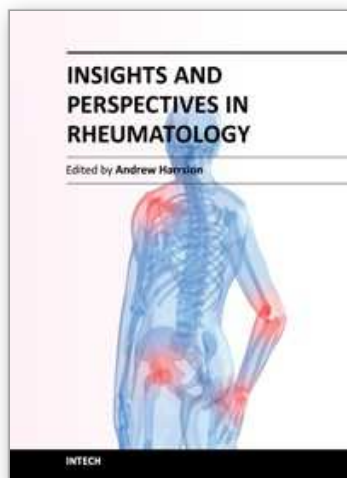
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