PhD thesis

Epitope mapping of natural antibodies

Dr. Tamás Czömpöly

Department of Immunolgy and Biotechnology
University of Pécs, Faculty of Medicine

Project leader: Prof. Dr. Péter Németh

Department of Immunolgy and Biotechnology
University of Pécs, Faculty of Medicine

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List of Abbreviations

APCs	Antigen presenting cells
BCR	B-cell receptor
CRP	C-reactive protein
CS	Mitochondrial citrate synthase
GST	Glutathione S-transferase
HBX	Hepatitis B virus X antigen
HBV	Hepatitis B virus
LPS	Lipopolysaccharide
MARCO	Macrophage receptor with collagenous structure
MBP	Mannose binding lectin
MDH	Malate dehydrogenase
MHC	Major histocompatibility complex
MMR	Macrophage mannose receptor
nAbs	Natural antibodies
nAAbs	Natural autoantibodies
PAMPs	Pathogen associated molecular patterns
PDH	Pyruvate dehydrogenase
PRRs	Pattern recognition receptors
RAG	Recombination activating gene
TCR	T-cell receptor
TLRs	Toll-like receptors
SAP	Serum amyloid protein
SLE	Systemic lupus erythematosus

Summary

The evolutionarily ancient innate immunity operates with non-clonally distributed promiscuous recognizing molecules. The later appeared adaptive recognizing system uses cells expressing clonally distributed highly variable antigen receptors.

Natural antibody (nAb) producing B-1 B cells are considered an intermediate stage of evolution between innate and adaptive immunity. nAbs are immunoglobulins that are produced without antigen priming. nAbs can recognize foreign targets and may serve in the first line of immune defense during an infection. Natural autoantibodies (nAAbs) present in the serum of both healthy humans and patients suffering from systemic autoimmune diseases recognize a set of evolutionarily conserved self-structures. Because of their endosymbiotic evolutionary origin, proteins compartmentalized into mitochondria represent an interesting transition from prokaryotic foreign (non-self) to essential (self) molecules.

In this work we have demonstrated the presence CS recognizing nAAbs. We investigated the possible overlap in recognized epitopes of innate and self-reactive nAbs and surveyed changes in physiological autoreactivity under pathological autoimmune conditions. In order to be able to perform the epitope mapping analysis we have set up a filamentous phage based random peptide library method with monoclonal antibodies recognizing the Hepatitis B virus X antigen and mouse CD45, respectively. We have built a lambda phage based CS antigen fragment library for further mapping.

Epitope mapping analysis of the mitochondrial inner membrane enzyme, citrate synthase (CS) (EC 2.3.3.1) by synthetic overlapping peptides and phage display libraries using sera from healthy individuals and from SLE patients revealed CS recognizing nAAbs with IgM isotype. Our results show that while there is no favored region of the CS molecule recognized exclusively either by healthy individuals or patients with SLE, the fine epitope pattern is different in the two groups examined. We analyzed cross reactive epitopes on human CS, bacterial CS, and various standard autoantigens. We have found three cross-reactive epitopes between human and bacterial CS, and we have identified a cross reactive antigen determinant on nucleosome antigen.

The anti-CS nAAbs by participating in the nAb network, could function in innate defense mechanisms and at the same time recognize a target antigen (nucleosome) in a systemic autoimmune disease. Thus, at the level of recognized epitopes there is a possible new link between the innate like component and the adaptive-autoimmune arm of the humoral immune system. We speculate that some nAbs in terms of their antigen recognizing characteristics are resembling the non-clonally distributed pattern recognition receptors of innate immunity.

Introduction

Microorganisms present in the environment continuously come into contact with the human body through external or internal epithelial surfaces. During evolution all multicellular organisms have developed defense mechanisms capable of eliminating these invading pathogens without causing damage to self structures. Consequently, discriminating self from non-self is of key importance for directing immune functions effectively operating on the basis of distinct recognition systems: innate and adaptive. These two arms differ from each other in several important features and their cooperation is essential for the correct function of immune defense. 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.

Innate immunity

Innate immunity serves as first line of defense against pathogens. Its early evolutionary appearance is indicated by its presence in all multicellular organisms including plants, invertebrates and vertebrates. Innate immunity uses receptors that are ancient in their evolutional 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 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. 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. (Table 1.) These molecules facilitate opsonisation for phagocytosis and aid the complement system in destroying pathogens which have been bound by these secreted proteins. The third functional group is constituted by signaling receptors such as the Toll-like receptors (TLRs) which activate several intracellular signaling cascades, eventually leading to the activation of many immune response genes.

PRR	Protein/domain family	Ligands
Cell surface, endocytic PRRs		
MMR	C-type lectin	Terminal mannose residues
CD14	Leucine rich repeats	LPS, peptidoglycan
SRs	Scavenger receptor cysteine rich domain	LPS, dsRNA, oxidized LDL
MARCO	Scavenger receptor cysteine rich domain	LPS, acetylated LDL, bacterial cell wall
Secreted PRRs		
MBP, C1q, SP-A, SP-D	C-type lectin	Bacterial and viral carbohydrates
CRP, SAP	Pentraxins	Phosphorylcholine
LBP	Lipid transfer protein family	LPS
Signaling PRRs		
TLR family	Leucine rich repeats/TIR domain	LPS, lipoteichoic acids, peptidoglycan, lipoarabinomannan, dsRNA, unmetylated CpG DNA, heat shock proteins, chromatin-Ig comlexes

Table 1. PRRs and ligands recognized by them.
MMR: macrophage mannose receptor, MARCO: macrophage receptor with collagenous structure, SAP: serum amyloid protein (adapted from Medzhitov 2001).

As it can be seen in Table 1., 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. Thus, the TLR family possibly represents an important milestone on the way to a recognition system characteristic for adaptive immunity.

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 defense 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 over an evolutionary time scale, and organisms possessing self reactive PRRs were eventually eliminated. This prevents autoimmunity in those organisms which have only the innate recognition system.

Adaptive Immunity

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 defense (T and B cells), appeared later during the evolution. It can be generally found in jawed vertebrates (Klein 1989), however the earliest species with a variable antigenreceptor based adaptive-like recognition system are jawless fishes (lamprey, hagfish). These fishes 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, Cannon et al. 2005, Pancer et al. 2005, Alder et al. 2005). The appearance of adaptive immune system in jawed vertebrates gives the impression of a "sudden" change between jawless and jawed fishes. This "big bang" hypothesis concerning gene duplication events, acquisition of a retrotransposon and the appearance of molecules such as major histocompatibilty complex, T- and B-cell receptors (Abi Rached et al. 1999, Kasahara et .al 2004) has recently been challenged by showing that integration of minor changes accumulated over an extended evolutionary time lead to the appearance of adaptive immune system (Klein and Nikolaidis 2005).

Whatever may have happened in the foggy past, the presence of adaptive immune system in highly developed and complex organisms provides them an additional level of protection and, consequently, a substantially inreased chance of survival. Since invertebrate species rely on innate defense 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. However, 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 orchestrated 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 (Oettinger et al. 1990). 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, in the past few years it was clearly demonstrated for B cell maturing in the bone marrow and spleen. (von Boehmer et al. 2003, Cancro and Kearney 2004). The ligand which 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 signaling 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 harboring allele could be rearranged (receptor editing), giving the cell a second chance to produce an antigen receptor unreactive with self structures above threshold (Edry and Melamed, 2004). 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)

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 costimulatory molecules on APC surface is critical for the activation of both T and B cells. In the absence of appropriate costimulation the activation signal remains below threshold level and the adaptive immune response will not be activated (Figure 1).

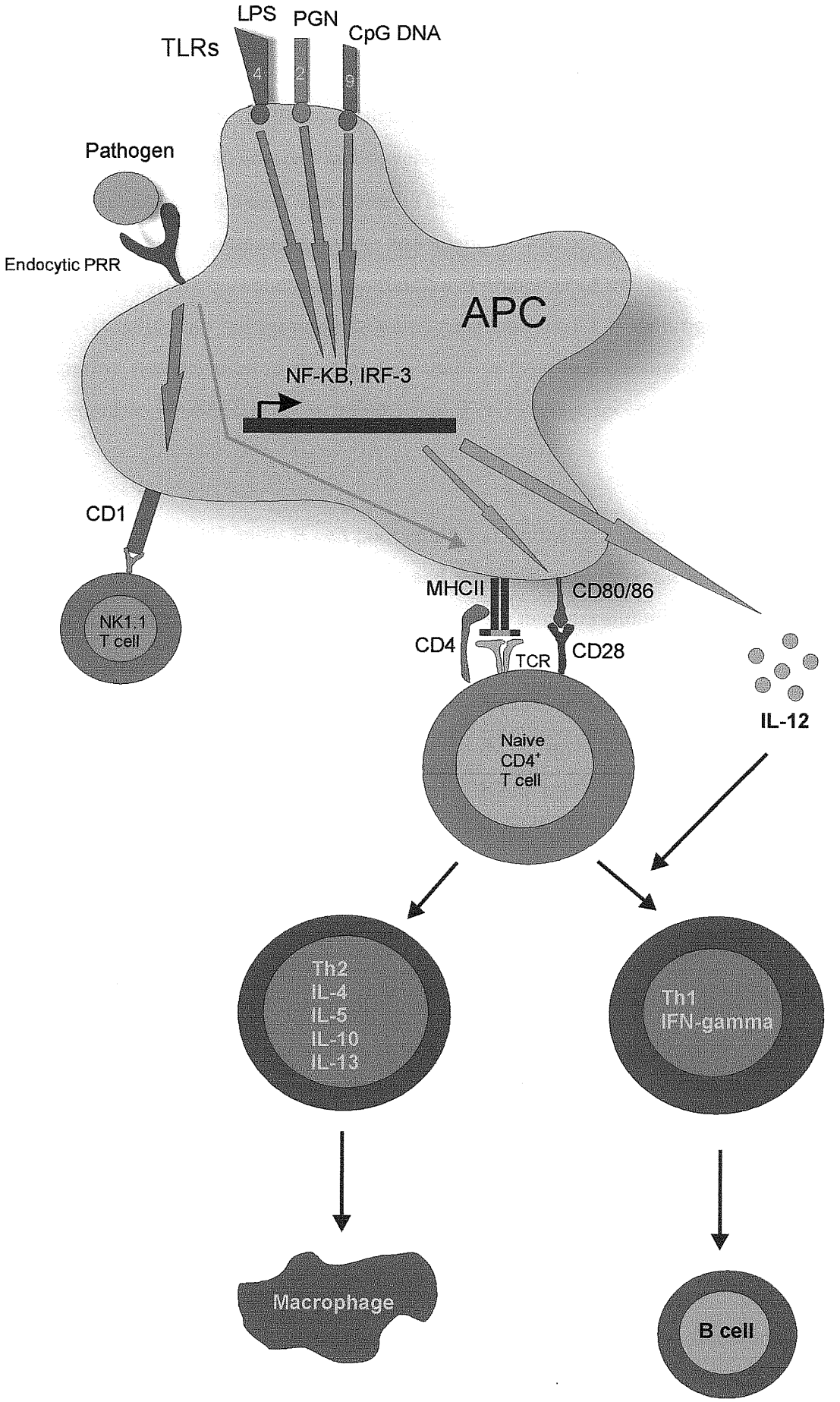


Figure 1. Pathogen recognition by innate recognizing molecules activates adaptive immunity. (adapted from Medzhitov 2001).

Natural immune system

A well defined immune machinery has recently been described between the innate and adaptive type immune systems. A distinct set of lymphocytes – both T and B cells – exists with characteristic phenotypes and specialized functions. B1 B cells and γδT cells studied intensively both in humans and mice. These subsets of cells exhibit common phenotypic characteristics and posses both innate and adaptive features, suggesting a transitional stage in the immune system's evolution. The functional character of antigen recognition by γδ T cells and B1 B 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.

B1 B cells

The peripheral naive B cell pool could be divided into three distinct subgroups: mature follicular B cells, marginal zone B cells and B1 B cells. In mice these groups are identifiable by the differential expression of certain cell surface markers summarized in Table 2.

Cell Type	Surface Phenotype
Mature follicular B cell	$B220^{hi}IgD^{hi}IgM^{lo}CD23^{+}CD21^{int}$
Marginal zone B cell	$B220^{+}IgD^{lo/-}IgM^{hi}CD23^{-}CD21^{hi}CD1d^{hi}$
B1a B cell	$B220^{+}IgD^{lo/-}IgM^{hi}CD11b(Mac\text{-}1)^{+}CD5^{+}$
B1b B cell	$B220^{+}IgD^{lo/-}IgM^{hi}CD11b(Mac\text{-}1)^{+}CD5^{-}$

Table 2. Cell surface markers characteristic for distinct B cell subgroups in mice.

Follicular B cells participate in T-dependent germinal center responses, while marginal zone B cell due to their specialized tissue localization respond to blood borne pathogens in a T-independent fashion. Since marginal zone B cells express high level of costimulatory molecules CD80 and CD86 they may also present blood borne antigens to naive T cells, thus they also might participate in T-dependent responses. In addition, they may also serve as antigen-transport cells into the follicles.

Originally, B1 B cells were distinguished from B2 cells on the basis of their expression of CD5, a glycoprotein marker previously considered to be T cell specific (Boyse et al. 1968, Ledbetter and Herzenberg 1979). CD5 is a type I transmembrane glycoprotein with three scavenger receptor cysteine rich domains and a highly conserved intracellular domain. Its role in signaling was extensively studied both in T and B cells (Goodnow et al. 1988, Burgess et al. 1992, Osman et al. 1993, Hippen et al. 2000,). As it is associated with antigen receptor signaling complexes, the CD5 molecule considered to be a negative regulator

of TCR and BCR signaling (Tarakhovsky et al. 1995, Bikah et al. 1996). 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 cell 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 (Marcos et al. 1989, Kroese et al. 1992). They are long lived in vitro, can be forced with phorbol esters to proliferate, and they could not be activated through BCR crosslinking (Hayakawa and Hardy 1988, Morris and Rothstein 1993, Rothstein and Kolber 1988). 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 fever N insertions than the repertoire of B2 cells (Kantor et al. 1997, Forster et al. 1988).

There is a long-standing dispute over the developmental origin of B1 B cells. According to the lineage model, B1 B cells are generated from fetal precursors present in the fetal liver, omentum and splanchnopleura (Herzenberg 2000, Solvason et al. 1991, Godin et al. 1993). 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 (Cong et al. 1991, Clarke and Arnold 1998). 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, Hayakawa et al. 1999, Arnold et al. 1994).

Functions of B1a cells include the participation in the early phases of immune responses (Martin et al. 2001, Wardemann et al. 2002, Baumgarth et al. 1999) and most importantly the production of natural antibodies, which is substantiated by the ability of B1 cells transferred adoptively into irradiated mice to restore normal IgM level (Herzenberg et al. 1986). 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.

Natural antibodies and natural autoantibodies

Natural antibodies (nAbs) are immunoglobulins mostly of IgM isotype, and are secreted by B1 cells without immunization with antigen (Hayakawa et al. 1984, Couthino et al. 1995). These antibodies can recognize genetically conserved sequences of pathogens and may serve in the first line of immune defense during an infection (Ochsenbein et al. 1999). In contrast, natural autoantibodies (nAAbs) present in the serum of both healthy humans and patients with systemic autoimmune diseases recognize a set of self-structures that have been conserved during evolution (Cohen and Young 1991). Most nAAbs belong to the IgM or IgG isotype (Avrameas 1991, Mouthon et al. 1995,), and show polyreactivity with a broad range of affinities for the recognized epitopes (Lacroix-Desmazes et al. 1998). Several functions have been suggested for nAAbs: they may participate in the selection of immune repertoires, play a role in the acceleration of primary immune responses (Ehrenstein et al. 1998), aid the clearance of apoptotic cells (Peng et al. 2005), possess anti-inflammatory effects (Miletic et al., 1996) and contribute to the maintenance of immune homeostasis (Lacroix-Desmazes et al. 1996). Discrimination of nAbs from nAAbs is somewhat artificial since given the limited B1 immunoglobulin gene repertoire driving natural antibody production and the numerous distinct antigens recognized (Table 3.) it is probable that specificities with self non-self cross reactivity exist.

Pathogen associated antigens	**Self structures**
Phosphoryl choline	Idiotypes, variable regions of antibodies and surface Ig on B cells
Phosphatidyl choline	Idiotype, framework and constant regions of T-cell receptors
LPS	CD4
Influenza Virus	CD5
Pneumococcal polysaccharides	MHC class I molecules
Heat shock proteins	Heat shock proteins
	Fcgamma receptors
	Autologous blood group antigens
	FVIII
	Cytoskeletal antigens: actin, titin

Table 3. Antigens recognized by natural antibodies. (Adapted from Lacroix-Desmazes et al., 1998)

Based on the above properties of natural antibodies, these molecules could be considered as the innate-like arm of humoral immune system.

Mitochondrial inner membrane enzymes

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 maintainence 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 nAAbs. In particular, little is known about the possible overlap between recognized epitopes of innate and self-reactive nAbs.

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 immonoreactivity with these molecules are less studied, and the possible changes in physiological autoreactivity under pathological autoimmune conditions remain largely unclear.

To address these issues we have chosen a mitochondrial inner membrane enzyme, citrate synthase (CS) (EC 2.3.3.1) as model antigen for epitope mapping using sera of healthy individuals and patients having various systemic autoimmune disease (systemic lupus erythematosus, rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyosits, systemic sclerosis, Raynaud syndrome and Sjörgen syndrome). The citrate synthase 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.

Aims of the presented work:

1. Setting up the bacteriophage displayed random peptide library method in our laboratory for epitope mapping

2. Detection of nAAbs in the sera of healthy individuals and patients with systemic autoimmune disease.

3. Affinity purification of anti-CS nAAbs and cross-reactivity testing with other mitochondrial inner membrane enzymes, with bacterial CS and with autoantigens targeted in autoimmune diseases.

4. Building of a CS antigen fragment library displayed on phage lambda.

5. Comparison of epitopes recognized by nAAbs in healthy individuals with those recognized in systemic autoimmune patients.

Methods

Patients and control sera

Serum samples from healthy individuals: 63 Hungarian blood donors from the Blood Transfusion Service of Baranya county, Pécs (age: 36.8 ± 17.1 years; 32 women, 31 men); a standardized panel from 51 British blood donors and 176 Finnish blood donors (by the courtesy of professor G. Füst and Z. Prohaszka, 3rd Department of Internal Medicine at the Semmelweis University, Budapest); 44 serum samples from healthy infants from the Pediatrics Clinic, University of Pécs (age: 11.5 ± 4.5 months; 26 females, 18 males) and samples of patients with systemic autoimmune diseases: 326 clinically well-documented cases of systemic lupus erythematosus (SLE), rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyosits, systemic sclerosis, Raynaud syndrome and Sjörgen syndrome from the Immunology and Rheumatology Clinic, University of Pécs (age: 44,2 ± 14,1 years; 284 women, 42 men) were used in this work with the permit of the Ethical Committee of the Medical Center of the University of Pécs.

Detection of mitochondrial enzyme specific autoantibodies by ELISA

96-well polystyrene plates (NUNC) were coated with CS, malate dehydrogenase (MDH; EC 1.1.1.37) and pyruvate dehydrogenase (PDH; EC 1.2.4.1) from porcine heart (Sigma) in 0.1M bicarbonate buffer, pH 9.6. Following the saturation of non-specific binding sites with 0.5% gelatin (Sigma) in PBS (pH 7.3), serum samples were incubated in triplicates at 1:100 dilutions in washing buffer (PBS, 0.05% Tween 20) for 60 min. Finally, the plate was incubated with HRPO conjugated anti-human-IgA, or -IgG or –IgM specific secondary antibody (Dako) for 60 min. The reaction was developed with *o*-phenylenediamine (Sigma), and measured on an iEMS MF microphotometer (ThermoLabsystem) at 492 nm. Cut off values of each groups examined were calculated from the average of measured OD492 data. Sera having higher OD value than average + 2SD were considered positive. All measurements were standardized with a monoclonal anti-citrate synthase antibody (Clone 4H3-E5) we produced previously (Nemeth P et al, 1991).

Affinity purification of sera on CS

CS from porcine heart was coupled to cyanogen-bromide activated sepharose 4B (Sigma) according to the manufacturer's instructions. Fifteen ml sera of 30 healthy blood donors and 14 patients with autoimmune disease were passed three times through the CS-sepharose resin. After washing antibodies were eluted in glycine-HCL pH 2.5, fractions were neutralized with

1 M TRIS and were tested for CS reactivity with indirect ELISA using HRPO conjugated anti-human-IgA, or -IgG or –IgM specific secondary antibody (Dako)

Cross reactivity testing of CS affinity purified sera

Cross reactivity with additional mitochondrial inner membrane enzymes was tested with indirect ELISA using MDH and PDH from porcine heart (Sigma) as antigens.

Reactivity with E.coli CS was tested with pin-bound overlapping decapeptides as described previously. (Petrohai et al., 2004; Nyarady et al., 2005)

Recognition of autoantigens implicated in various autoimmune diseases were tested using indirect ELISA kits developed for the quantitative measurement of double stranded DNA, nucleosome, Cenp-B, MPO, PR3, alpha-fodrin, gastric parietal cell, intrinsic factor, Asca, gliadin, tissue transglutaminase, cardiolipin, β2-glycoprotein-1, phosphatidyl serine, prothrombin (Orgentech) and Sm, RNP, SSA, SSB, Scl-70, Jo-1, CCP, thyreoglobulin, glomerular basal membrane (Hycor) specific autoantibodies.

Immunocytochemistry

Immunocytochemistry with affinity purified anti-CS sera from SLE patients was performed on Hep-2 cells using the Anafluor kit (Diasorin). In brief, slides were incubated with sera diluted 1:100 in PBS for 30 min. Following 3x5 min wash in PBS rabbit anti-human IgM secondary antibody (1:100, Dako) was added and incubated for 30 min. After washing the bound antibodies were visualized with a goat anti-rabbit FITC conjugate (Sigma).

Random peptide library screening

The filamentous phage library displaying cyclic nine amino acid random peptides as a fusion to the N-terminal of the M13 major coat proteinVIII was constructed previously (Felici et al.,1991). In order to set up the method, affinity selection of the library was initially performed with two of our monoclonal antibodies (mAb), produced against the hepatitis B virus X antigen (HBX) and the mouse CD45 molecule (IBL-8 mAb). Then affinity selection of phages with CS affinity purified sera from SLE patients was performed using the biopanning technique (Parmley and Smith, 1988). In brief, microtiter plates were coated with mAbs or affinity purified anti-CS sera (40 μg/ml during the first and 4 μg/ml during the second and third rounds of panning). After washing with PBS/0.05%Tween-20 and blocking with a solution containing PBS/3%BSA, 10^{10} ampicillin transducing unit (ATU) phage (blocked with PBS/1%BSA) was added and incubated for 2h at room temperature. The plate was washed with PBS/Tween-20 (0.05% in the first or 0.5% in the second an third round of panning) and the bound phage were eluted with 1mg/ml BSA /0.1 M glycine pH 2.2.

Following neutralization with 2M TRIS, 10 ml XL1-Blue ($O.D._{600}$:0.5) was infected and plated on LB agar plates containing 50 μg/ml ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with 10^{11} plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37°C, phage were precipitated with 16.7% PEG8000/3.3 M NaCl twice and resuspended in TBS. Enrichment was monitored by indirect ELISA with phage pools after each selection step. Following the third round of panning, randomly chosen clones were picked up and tested for reactivity with the selecting sera by indirect ELISA. Based on the ELISA results forty clones were selected for DNA sequencing.

Construction of recombinant overlapping HBX fragments

For the production of recombinant overlapping HBX fragments we used the glutathione S-transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech). Three overlapping parts of the HBX gene (subtype ayw, Bichko et al. 1985) were amplified by PCR using primers with BamHI and EcoRI restriction sites. The fragments were cloned into the expression vector pGEX-6P-1. Each construct was verified by sequencing. Fusion proteins were expressed in E. coli DH5-alfa, and the recombinant proteins were purified using the GST-glutathione affinity system with modifications according to Marczinovits et al. 1997.

ELISA with overlapping HBX fragments

Microtiter plates (Nunc) were coated with 0.1μg antigen in 0.1M bicarbonate buffer, pH 9.6, at 4°C overnight. PBS-gelatin was used to block the free binding sites. The anti-HBX Mab was used at final concentration of 1 μg/ml for 1 hour at 37°C. Reactions were developed by a HRPO labeled anti-mouse IgG secondary antibody.

Flow cytometry

The cells from lymph nodes were released between the frosted ends of two glass slides into chilled PBS containing 0.1% BSA and Na-azide. The cells were filtered through a cell strainer, and were collected by centrifugation at 800 g for 10 min at 4 °C. The pellet was resuspended in cold PBS-BSA, and adjusted to $5x10^6$ cells/ml. The inhibitory effect of phages displaying the putative IBL-8 epitope was determined using phages purified with PEG precipitation. The phages (10^{12}/ml) were incubated with 1 μg/ml purified mAb in the final volume of 100 μl for 30 min at room temperature, then to the phage-mAb mixture 50 μl lymph node cell suspension was added, prepared as above. After 20 mins on ice, the cells were washed, and incubated with phycoerythrin-conjugated goat anti-rat IgG (BD Pharmingen). The reaction was stopped by repeated washing, the cells were fixed, and the

samples were analysed by flow cytometry. Controls included anti-CD45RA/B220 mAb in place of IBL-8 mAb, and M13 phages replacing the IBL-8 epitope expressing phages.

Construction of a CS antigen fragment library

Total RNA was isolated using TriReagent (Sigma) from $3x10^6$ mononuclear cells obtained by Ficoll Paque (AmershamPharmacia) gradient centrifugation from peripheral blood of a healthy blood donor. 5 μg total RNA was reverse transcribed with Superscript II RT (Invitrogen) according to the manufacturer's instructions. cDNA encoding for the full length human mitochondrial citrate synthase was amplified with the following primers: 5'-ATGGCTTTACTTACTGCGGC-3' and 5'-TTACCCTGACTTAGAGTCCAC-3'. The PCR reaction contained 300mM of each dNTP, 1.5 mM $MgSO_4$, 1 μM of each primer, 5 μl cDNA and 5 units of ProofStart DNA polymerase (Qiagen) in a 100 μl final volume, cycling was done with the following profile: 95 C 5min, 35 cycles of 95 C 1min, 51 C 30s, 72 C 2min, final extension at 72 C for 10 min. The PCR product was separated on a 1.5% agarose gel and purified using the Quiaquick Gel Extraction Kit (Qiagen). Following A-addition it was cloned into a T/A vector using the InsT/Aclone PCR Product Cloning Kit (Fermentas). The identity of insert was verified by sequencing on an ABI3100 Avant genetic analyzer.

Library construction was done using the lambdaD-bio phage display vector (a kind gift from Dr. Alessandra Luzzago; Instituto di Ricerche di Biologia Molecolare, Italy) as described (Ansuini et al., 2002). In brief, inserts were produced by tagged random primed elongation and amplification using SpeI and NotI tagged random primers (Santi et al., 2000) and CS cDNA as template excised with BamHI and EcoRI (Promega) digestion from the plasmid mentioned above. Following purification with the Quiaquick PCR purification kit (Qiagen) and size selection on Wizard columns (Promega) inserts were digested with SpeI and NotI (Promega). Twenty ligations were set up containing 1 μg of SpeI/NotI digested lambdaD-bio DNA, 25 ng of SpeI/NotI digested insert, 30U of T4 DNA ligase (Fermentas) in a final volume of 5μl and incubated 48 hours at 4C . The ligation mixture was phenol-chloroform extracted, ethanol precipitated and packaged with the Ready To Go Lambda Packaging Kit (AmershamPharmacia). Phage were amplified by infecting log phase E.coli BB4 cells and plating them on LB agar plates. After plaque formation phage were eluted by an overnight incubation in SM buffer (100mM NaCl, 8.1 mM $MgSO_4$, 50mM Tris-HCl pH 7.5), concentrated with polyethylene glycol precipitation and resuspended in SM buffer supplemented with Complete EDTA Free Proteasae Inhibitor Cocktail (Roche).

Affinity selection of CS antigen fragment library

Affinity selection of CS antigen fragment library with CS affinity purified sera was performed essentially as described (Santini et al., 1998). Briefly, microtiter plates were coated with affinity purified anti-CS sera or anti-CS mAb 4H3E5 (developed in our lab) at 10 μg/ml in coating buffer. After blocking 10^{10} phage were incubated for 2 h at room temperature. Wells were washed five times and bound phages were recovered by in well infection of E.coli BB4 cells. The infected bacteria were plated on LB agar plates and phage were eluted then concentrated as described above. The affinity selection was repeated one more time and individual clones were picked up for DNA sequencing.

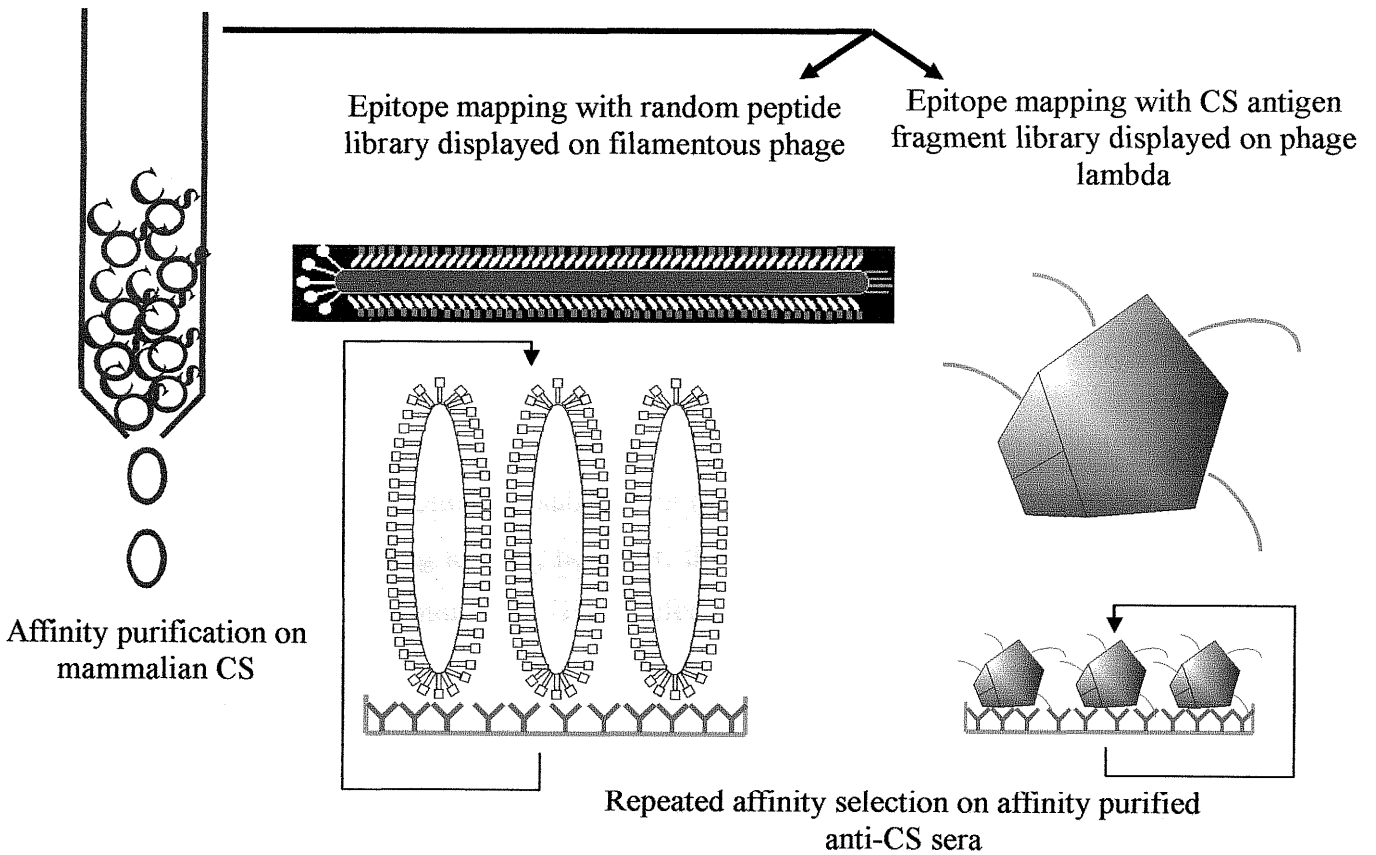


Figure 2. Illustration of techniques used for epitope mapping.

Results and Discussion

Introduction of the phage displayed random peptide library technique

In order to set up and optimize the phage displayed random peptide library method, affinity selection of the library was initially performed with our two mAbs, produced against the mouse CD45 molecule and the hepatitis B virus X antigen (HBX), respectively.

CD45, a transmembrane phosphotyrosine phosphatase, is amongst the most abundant glycoproteins displayed by leukocytes (Thomas 1989, Hermiston et al. 2003). The alternative splicing and variable expression of the exons near to the N-terminus of the molecule result in distinct extracellular isoforms expressed by cells with different functional and developmental properties, which are heavily glycosilated. Our aim was to map the epitope of our new rat monoclonal antibody IBL-8 against the exon C and to define the expression pattern of CD45RC isoform in mature and immature mouse B cells by using IBL-8. Among these 20 clones we have identified two types of clones displaying slightly different nonapeptides. A comparison of the deduced amino acid sequence of these two groups of clones with the primary sequence of the CD45 molecule assigns the epitope recognised by the IBL-8 mAb to amino acids 136–144 (ADTAFPVDT) (Table 4). This sequence lies within the exon C of the mouse CD45 molecule. Preincubation with both cA3 and cB6 phages as prototypes for the two groups completely inhibited the subsequent recognition of native antigen by IBL-8 mAb (Figure 3). It was interesting to note, however, that the same phages exerted only a reduced inhibitory effect for the binding of GL24 reference mAb to CD45RC, indicating that its epitope specificity is slightly different from that of IBL-8.

	Amino acids	Number of independent isolations
Mouse CD45 C domain	**PGERTVPGTIPADTAFPVDTPSLARN**	
CA3 phage clone	**AYTAFPLD**	**13**
CB6 phage clone	**NTAFPSGTS**	**7**

Table 4. Alignment of the amino acid sequence of CD45RC exon to the IBL-8 phage epitopes. The amino acid sequence spanning the 125–150 region of murine CD45 as a reference and the nonapeptide sequences of peptides displayed are shown. The consensus amino acids between the region 136 and 144 are underlined. The numbers of independent isolates refer to the clones sequenced and are found identical for the two distinct groups.

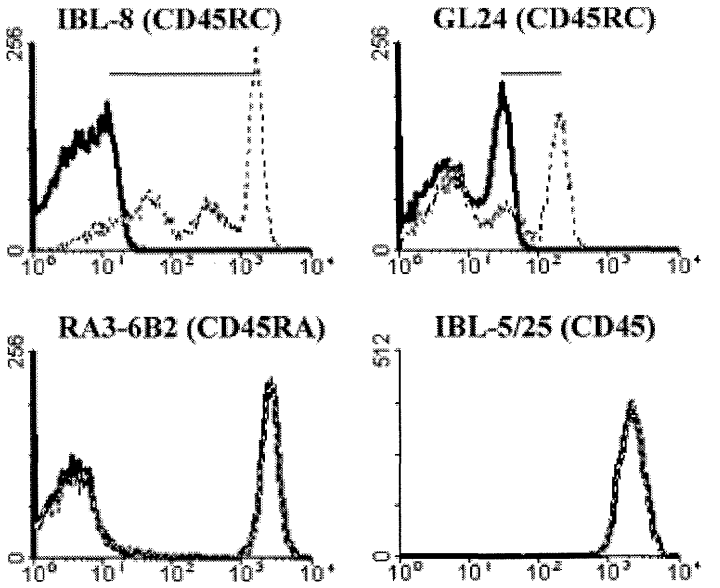


Figure 3. Specific inhibitory effect of phages displaying the IBL-8 epitope. cA3 phages were reacted with various anti-CD45 monoclonal antibodies (indicated at the top of each plot) followed by adding the phage–mAb mixture to mouse lymph node cells. The mAb binding was determined using PE-conjugated anti-rat IgG. Bold curves represent the samples with cA3 phage, the dashed curves illustrate the control labelling following preincubation with "empty" M13 phages. The horizontal lines in the anti-CD45RC mAb panels indicate the shift of peak channels of fluorescence.

We developed a set of mAbs against the Hepatitis B virus X antigen (HBX) for research and diagnostic use (Pal et al. 2001). We used a recombinant HBX protein fused to glutathione S-transferase (GST) as antigen for immunization and for subsequent testing. The fine epitope specificity of the newly generated monoclonal antibodies were unknown. However, the precise information about the epitope specificity of these antibodies was important for the further practical applications.

Hepatitis B virus (HBV) is an important etiologic agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. (Szmuness 1978). The smallest open reading frame of HBV, called X, encodes a 17 kDa protein of 154 amino acid with highly hydrophobic and disulfide-bonded characters (Caselmann 1996, Gupta et al. 1993). The X protein (HBX) is a multifunctional regulatory protein which modulates transcription, cell responses to genotoxic stress, protein degradation, and signaling pathways, but the precise function of HBX is not well understood (Andrisani and Barnabas 1999, Murakami 2001).

The determination of fine epitope structure recognized by one of our anti-HBX mAb became necessary due to the results of our retrospective study on human liver biopsy specimens. Moreover, for the quantitative measurement of the HBX from human serum, we developed a sandwich type ELISA which required a pair of anti-HBX mAbs with no overlapping epitopes, thus further necessitating the characterization of epiptopes of anti-HBX reagents employed in the assay.

P F L P K V L H K *
R M L P A S L H L
R R L P E E L H R
A R L P P S L H L
R N L P R A L S P
R R L P S A F H P
L R S L H L V R G
S R P P S L F R P
R N V F L I R T E
G R V F F L V R P
D R N I Y L V R Q

Table 5. Deduced amino acid sequences of clones selected with anti-HBX mAb from a library of nine amino acid random peptides displayed on filamentous phage.
* aminoacids 86.-94. of HBX

Based on immunoscreening and ELISA tests, we chose ten phage clones for DNA sequencing. A comparison of the deduced amino acid sequences of these clones revealed a consensus sequence of LPxxLH (Table 5.). This sequence can be found in the primary structure of HBX (amino acids 88.-93.). This is in agreement with our earlier Western-blot result performed with recombinant HBX antigen under reducing conditions (Pal et al. 2001), which indicated the linear type of epitope..

In order to confirm the result obtained by random peptide library, we constructed three overlapping GST fusion peptides spanning amino acids 77-142. of the HBX. The anti-HBX Mab recognized only the segment representing amino acids 77-116. No reaction was found when peptides containing sequence 96-135. and 116-142. were tested. According to the alignment of recombinant HBX segments only the sequence 77-95. could possibly contain the epitope bound by the anti-HBX Mab. (Table 6.) This calculation has verified the results of the random peptide library screen.

Fragment	AA	ELISA
METTVKAQPFLPKVLHKRTLGLSVMSTTDLEAYFKDCLF	77-116.	+
TLGLSVMSTTDLEAYFKDCLFKDWEELGEEIRLKVFVLGG	96-135.	-
FKDWEELGEEIRLKVFVLGGCRHKLVC	116-142.	-

Table 6. Overlapping recombinant HBX fragments. AA: corresponding aminoacids of the HBX protein; ELISA: reactivity with the anti-HBX Mab in ELISA .

In summary, we employed the screening of a random peptide library displayed on filamentous phage for epitope mapping of two of our mAbs. The method proved to be a good approach for the mapping of linear epitopes both on a cell surface molecule (CD45) and on a protein with special physicochemical properties (HBX).

Anti-mitochondrial enzyme specific antibodies in healthy individuals and systemic autoimmune patients

Using simple binding ELISA we demonstrated the presence of antibodies recognizing CS, MDH, and PDC in the sera of both healthy individuals and systemic autoimmune patients. Isotype-specific ELISA showed that enzyme-specific antibodies with IgM isotype are more frequently present in all investigated groups than those of IgG or IgA isotypes (Table 7.). No differences were found among the subgroups of healthy individuals; however, the incidence of anti-CS and anti-MDH autoantibodies with IgM isotype was significantly higher in autoimmune patients compared to the healthy controls.

We continued our investigations with CS specific IgM autoantibodies, as this group showed the most characteristic pattern of distribution. We followed the titer of anti-CS IgM antibodies in 53 healthy individuals selected from British and Hungarian blood donors with repeated sample collection minimum 3 times over a 5 years period. We found that the CS reactivity of individual sera remained permanently constant over this period (Figure 4).

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 nAAb repertoire established early in postnatal life (Mouthon et al., 1995, Stahl et al., 2000).

	CS IgA	CS IgG	CS IgM	MDH IgA	MDH IgG	MDH IgM	PDH IgA	PDH IgG	PDH IgM
Hungarian blood-donors (63)	4%	3%	10%	3%	2%	8%	3%	3%	10%
British blood-donors (51)	5%	4%	12%	3%	3%	9%	3%	4%	10%
Finnish blood-donors (176)	5%	3%	9%	2%	2%	8%	3%	5%	10%
Healthy infants (44)	2%	2%	7%	4%	4%	9%	2%	4%	9%
Systemic autoimmune patients (326)	5%	3%	24%	4%	4%	16%	4%	4%	6%

Table 7. Prevalence of mitochondrial inner membrane enzyme specific antibodies in human sera

Sera were tested for citrate synthase (CS), malate dehydrogenase (MDH) and pyruvate dehydrogenase (PDH) reactivity with isotype specific indirect ELISA. Values in parentheses indicate the number of individual serum samples tested.

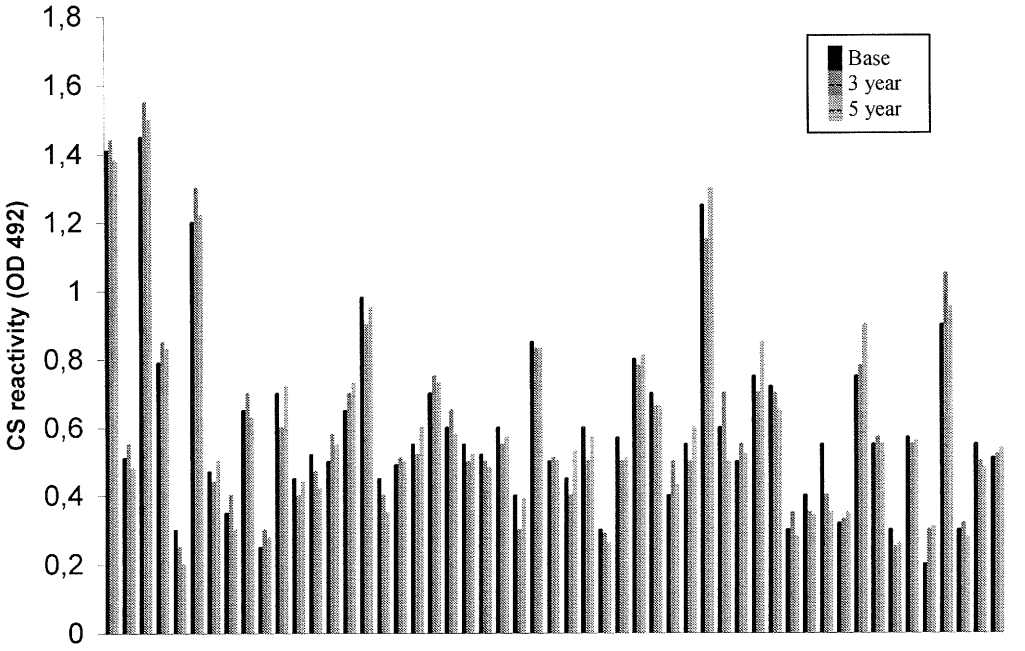


Figure 4. CS reactivity in healthy individuals over a five year period.
CS reactivity of sera from 53 healthy individuals was followed up during a five year period with IgM isotype specific indirect ELISA.

Affinity purification and cross reactivity testing of CS reactive sera

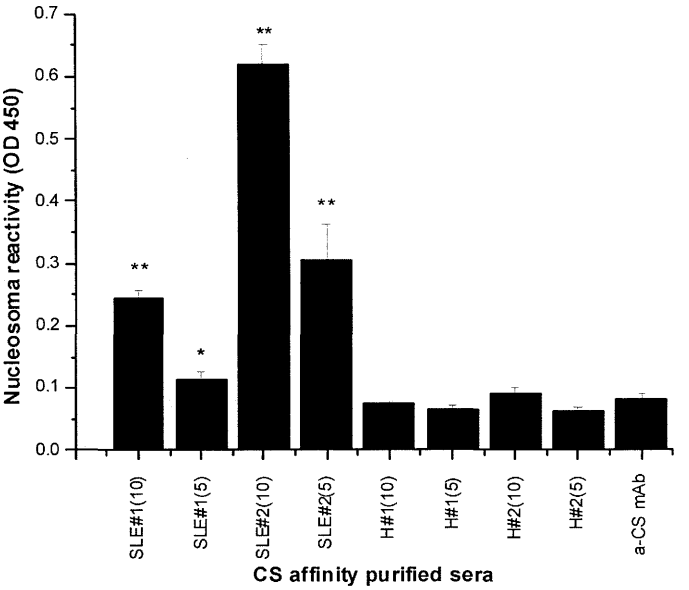
To exclude the masking effects of nonspecific bindings we purified anti-CS antibodies from 44 human sera (30 healthy and 14 autoimmune patients: 9 with SLE, 3 with systemic sclerosis and 2 with rheumatoid arthritis) by affinity chromatography for further experiments. Affinity purification was successful only in those cases (2 healthy and 2 SLE patients) where the actual serum had extraordinary high (OD492 >1.5) anti-CS reactivity. The eluted anti-CS antibodies from such sera were exclusively with IgM isotypes (data not shown).
Cross-reactivity of the affinity purified anti-CS antibodies with other mitochondrial inner membrane enzymes (MDH and PDC) was tested by indirect ELISA. The affinity-purified anti-CS antibodies did not recognize any of these antigens (data not shown).

Since previous studies have suggested that natural antibodies play an important role in the innate like component of the humoral immune response (Martin et al., 2001, Wardemann et al., 2002, Baumgarth et al., 1999), we investigated the possible overlap in nAAb recognized epitopes on mammalian and bacterial CS. Due to the prokaryotic origin of mitochondria, CS represents an attractive target molecule to examine the self-reactive nAAbs' capability to recognize epitopes on the foreign counterpart of the same molecule. To achieve this, we used sera affinity purified on mammalian CS for epitope mapping on CS from E.coli, using the overlapping synthetic peptide method. Only three cross reacting sequences were found: amino acids 124-133: FRRDSHPMAV (identity with human CS: 40%, similarity: 60%); amino acids 174-183: MCYKYSIGQP (identity with human CS: 30%, similarity: 40%) and amino acids

351-360: YFIEKKLYPN (identity with human CS: 40%, similarity: 60%), respectively. The three recognized sequences show only a limited homology with human CS, even though identical amino acids with a possible anchor function are present at corresponding positions. These amino acids contain either polar or charged side chains, which is in agreement with previous reports about the preferential amino acid composition of nAAb epitopes (Novick et al., 1992, Pashov et al., 2002). The three peptides, according to the three dimensional model, are located on the surface of the molecule. Moreover, two of the peptides (124-133 and 174-183), though separated by 50 amino acids in the primary sequence, are in close proximity on the structural model of folded protein, indicating that they represent the same antigenic region.

In order to examine the cross-reactivity of affinity-purified anti-CS sera on autoantigens attributed with a role in various autoimmune diseases, we performed several indirect ELISAs with commercially available autoantibody kits (see methods for the listing of antigens tested). The affinity-purified anti-CS sera from two SLE patients recognized nucleosome antigen (Figure 5A). To exclude the possibility of CS contamination in the nucleosome antigen preparation, we used our anti-citrate synthase mAb 4H3E5 (Nemeth et al, 1991) in the same ELISA system. For further verification of our results we carried out competition ELISA experiments using CS from porcine heart as competitor (Figure 5B).

A



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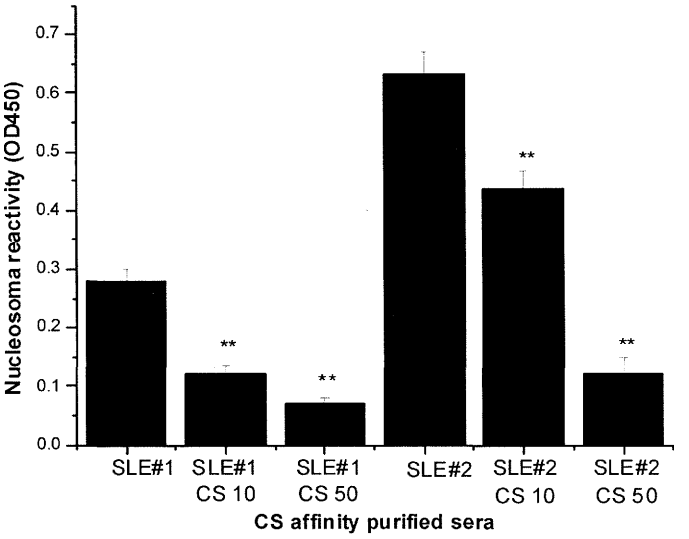


Figure 5. Anti-CS antibodies from SLE patients recognize nucleosoma antigen.
A: Affinity purified anti-CS sera from two SLE patients (SLE) and two healthy (H) individuals were tested for nucleosome reactivity. Numbers in parentheses indicate the protein concentration in μg/ml. CS contamination of the nuclesome antigen was controlled with an anti-CS mAb 4H3E5 (a-CS mAb).
B: Competitive ELISA with CS: Affinity purified anti-CS sera from two SLE patients (10 μg/ml) were preincubated with the indicated amount of CS (μg/100μl) for 1h then reactivity for nucleosome antigen was tested. All measurements were performed in triplicates and were repeated in three independent experiments. * $p < 0.05$, ** $p < 0.001$

In order to obtain further support for these findings we screened 46 additional sera from SLE patients for CS and nucleosome reactivity and performed CS affinity purification from the 11 double positive (high CS and nucleosome reactivity) patients' sera. All of the above mentioned 11 CS affinity-purified sera recognized the nucleosome antigen, which reactivity could be inhibited in competition with CS (mean nucleosome reactivity OD (450): 0.38 ± 0.15 without competition; 0.21 ± 0.09 in competition with 10 μg CS and 0.08 ± 0.03 in competition with 50 μg CS). In addition we performed fluorescent immunocytochemistry on Hep-2 cells. All of the CS affinity purified SLE patients's sera resulted a low-intermediate staining intensity in the nucleus - the nucleoli of the nuclei were consequently negative - characteristic for the recognition of nucleosomes. In addition we found an intermediate-high staining intensity in the cytoplasm, characteristic for the recognition of CS A representative image is presented in Figure 6. Our results show that there is indeed a cross-reactivity of anti-CS antibodies from SLE patients with nucleosome antigen.

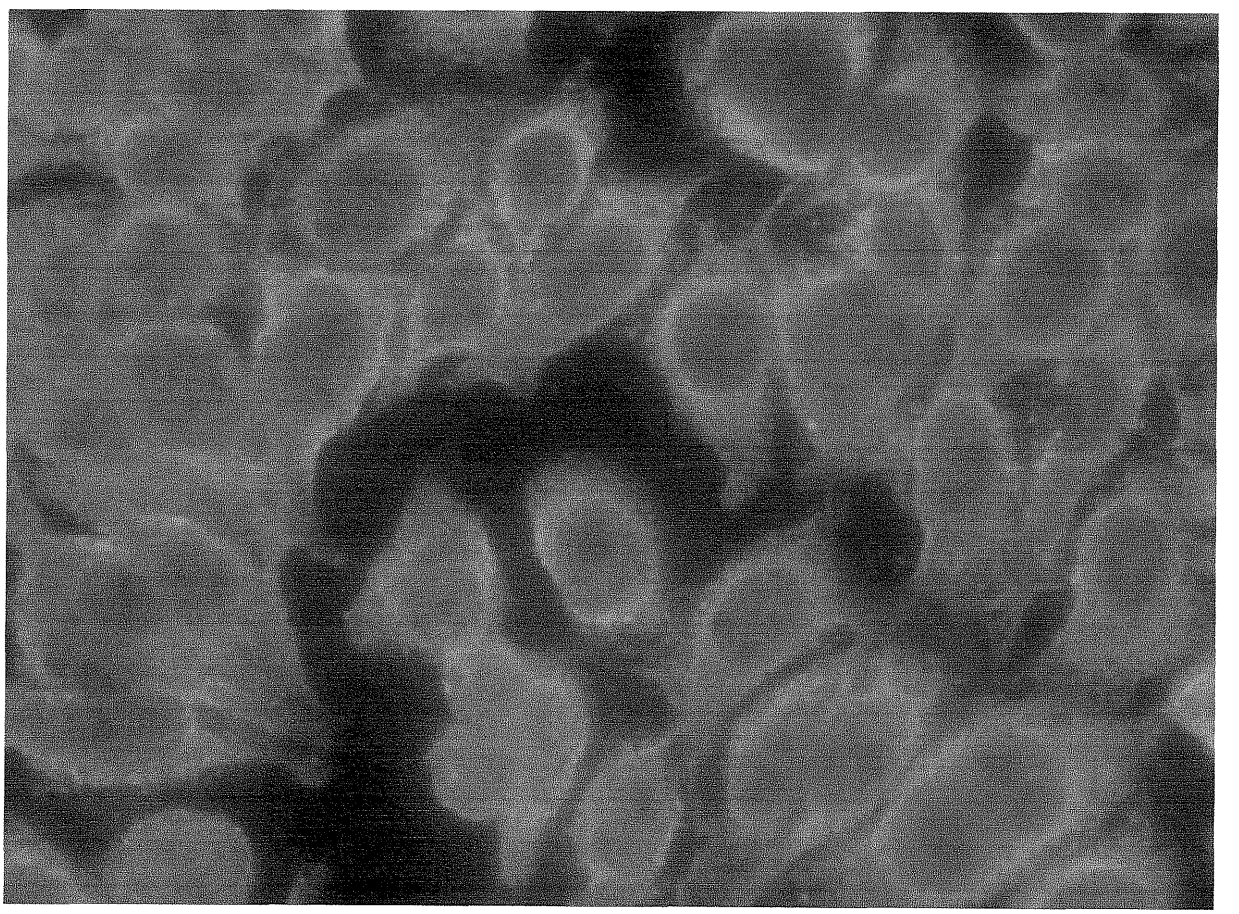


Figure 6. A representative image of immunocytochemistry performed on Hep-2 cells with CS affinity purified sera from SLE patients.

CS antigen fragment library construction

Epitope mapping with overlapping synthetic peptides is a widely used technique (Maeji et al., 1995, Uray et al., 2003), but its constraints include the uncertainties linked to in silico B-cell epitope prediction used for selection of antigenic regions (Nyarady et al., 2005), 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 (Scott and Smith, 1990, McCafferty et al., 1990, Marks et al., 1991, Farilla et.al., 2002). 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. 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.

For analysis of fine epitope structure of anti-CS autoantibodies we constructed a CS antigen fragment library displayed on bacteriophage lambda. The library contains approximately 10^7 insert bearing independent clones. First we tested the library by performing an affinity selection with the anti-CS mAb 4H3E5. After the second round of affinity selection 30 clones were chosen for DNA sequencing. Among them 4 distinct sequences were found repeatedly (Table 8.). These sequences could be aligned with amino acids 7-65. of human CS and the minimal epitope of our anti-CS mAb could be restricted to amino acids 31-59.

Number of independent isolations	Deduced amino acid sequence
5	AARLLGTKNASCLVLAARHASASS**TNLKDILADLIPKEQARIK TFRQQHGKTV**VGQITV
8	**TNLKDILADLIPKEQARIKTFRQQHGKTV**
9	ASCLVLAARHASASS**TNLKDILADLIPKEQARIKTFRQQHGK TVV**G
6	ASCLVLAARHASASS**TNLKDILADLIPKEQARIKTFRQQHGK TV**
huCS	AARLLGTKNASCLVLAARHASASS**TNLKDILADLIPKEQARIK TFRQQHGKTV**VGQITV

Table 8. Alignment of anti-CS mAb selected phage clones with human CS.
The number of independent isolations refers to the clones sequenced and found identical for the four distinct inserts. huCS indicates amino acids 7-65. of human CS.

Affinity selection of CS antigen fragment library with CS purified sera

Having demonstrated the effectiveness of our phage display CS antigen fragment library for epitope mapping, we proceeded to the epitope mapping of affinity-purified anti-CS sera. Following two rounds of affinity selection 20 clones selected with each serum were picked up for DNA sequencing. In contrast to the selection with our anti-CS mAb, these clones carry short peptide sequences which could also be aligned to human CS (Figure 7). These short sequences are scattered throughout the human CS sequence and it seems that practically the same regions of the molecule are recognized by the two groups of sera. According to our results obtained with phage displayed antigen fragments, while there is no favored region of the CS molecule recognized exclusively either by healthy individuals or patients with SLE, the fine epitope pattern is different in the two groups examined.

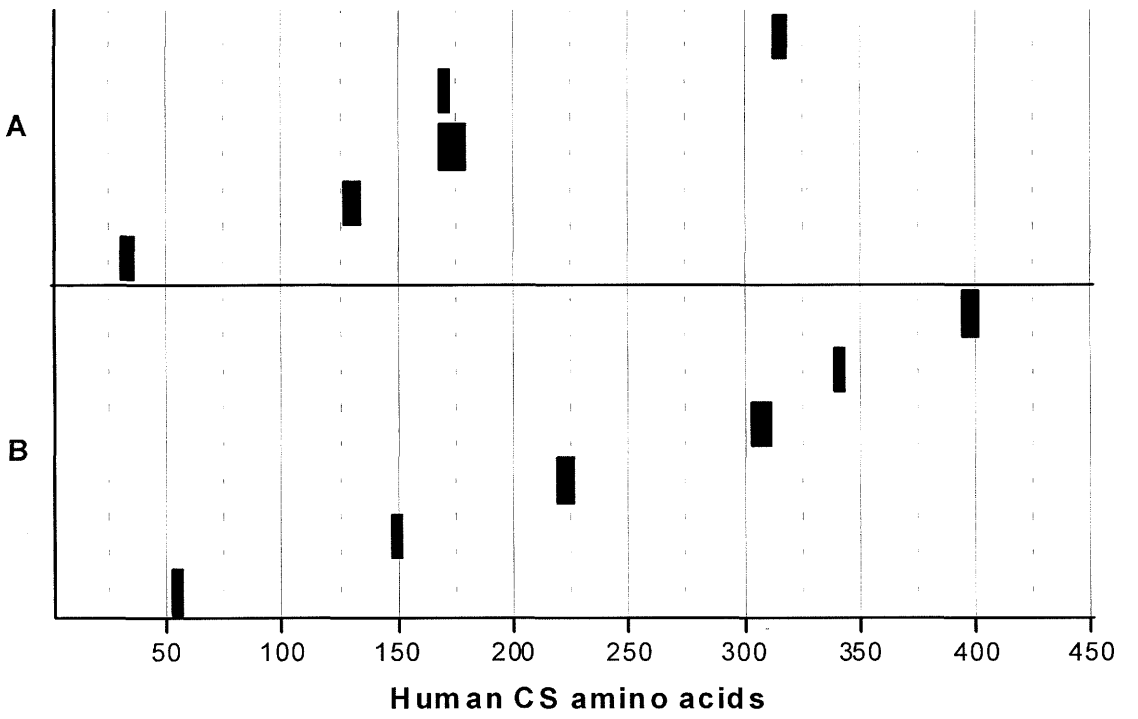


Figure 7. Alignment of anti-CS sera selected phage clones with human CS.
Deduced amino acid sequences of phage clones selected with affinity purified anti-CS sera prepared from healthy individuals (A) and patients with SLE (B) are plotted along the human CS sequence.

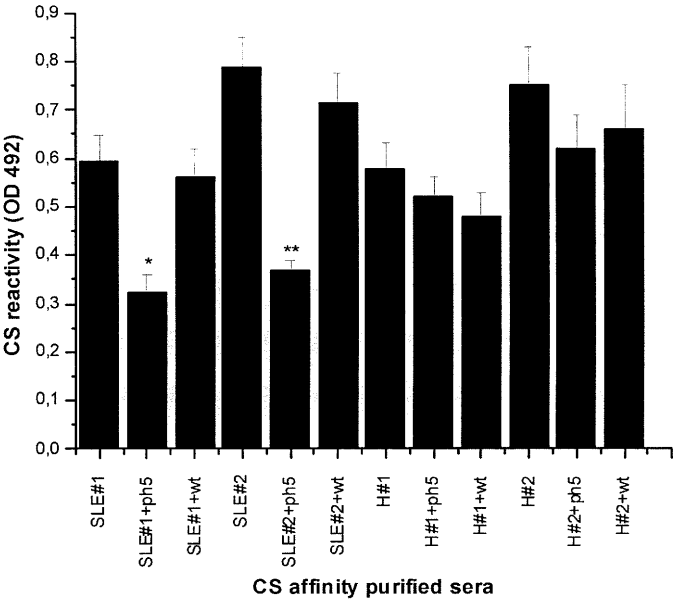
Random peptide library screening with CS purified sera

The unexpected cross-reactivity of SLE patients' CS affinity-purified sera with nucleosome antigen could not have been unequivocally explained by the results of epitope mapping performed either by the synthetic overlapping peptide or phage displayed antigen fragment method. Therefore we screened a nine amino acid random peptide library with CS affinity purified sera from two patients with SLE. In this system random peptides are presented at high copy number, making the identification of low affinity interactions easier. The 40 sequenced clones carry similar peptides which show partial homology with human CS (Table 9.) We isolated a phage clone (YAAPSHQSH phage#5) which carries a peptide corresponding to amino acids 145-150. of human CS and performed competition ELISA with it both for CS and nucleosome antigen.(Figure 8.). According to our results, phage#5 inhibited the CS reactivity of CS affinity purified sera from SLE patients but not from healthy individuals. When tested for blocking nucleosome reactivity, phage#5 proved to be an efficient inhibitor of the nucleosome reactivity measured with CS affinity purified sera from SLE patients. These results indicate that the CS-nucleosome cross-reactivity is at least partly caused by antibodies recognizing the CS epitope (amino acids 145-150.) mimicked by phage#5.

Number of Independent Isolations	Deduced amino acid sequence
2	APASPYQCG
1	APASPYQSG
5	EAASPYQSH
5	YAAPSSQSH
9	YAAPSRQSH
18	YAAPSHQSH
huCS 145-150	AALPSH

Table 9. Alignment of CS affinity purified sera selected random peptides with human CS. The number of independent isolations refers to the clones sequenced and found identical for the given peptide. huCS indicates amino acids 145-150. of human CS.

A



B

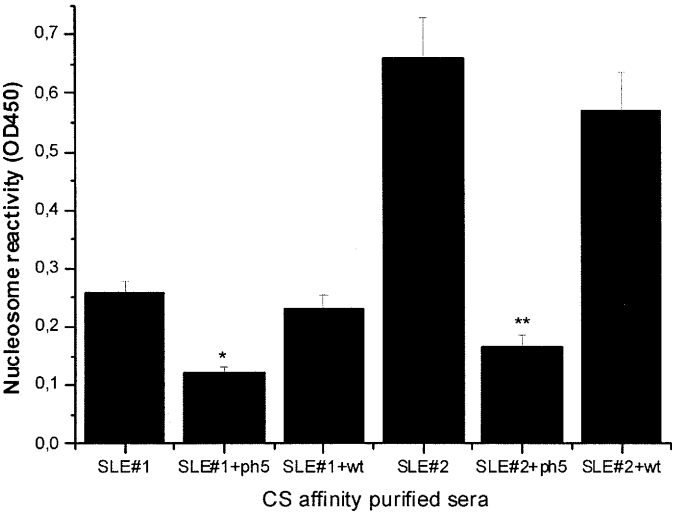


Figure 8. Competition for CS and nucleosome antigen with a phage clone selected from a random peptide library. Competition ELISA was performed with phage#5 (YAAPSHQSH), selected with CS affinity purified SLE patients' sera from a random peptide library. A: CS affinity purified sera from patients with SLE or healthy individuals (SLE#1, #2 and H#1, #2 at 1 μg/ml) were preincubated with 10^{10} phage#5 (ph5) or wild type (wt) particles for 1h at 4°C, then reactivity for CS was measured by indirect ELISA. B: CS affinity purified sera from patients with SLE (SLE#1, and #2 at 10 μg/ml) were preincubated with 10^{10} phage#5 (ph5) or wild type (wt) particles for 1h at 4°C, then reactivity for nucleosome antigen was measured by indirect ELISA. * $p < 0.05$, ** $p < 0.005$

Reviewing our data obtained by phage displayed CS antigen fragment library revealed that a lambda phage clone, carrying practically the same part of the CS molecule as the filamentous phage clone #5, was isolated with the SLE patients' sera. Therefore, on the basis of evidence substantiated by the random peptide and CS antigen fragment library methods, we conclude that this CS epitope (145-150.) is preferentially recognized by the SLE patients' sera. The cross reactive epitope identified on human CS (145-150.) is located on the surface of the molecule. It is interesting to note that it is part of the region which contains two of the E.coli CS cross reactive determinants (124-133 and 174-183). We hypothesize that this (124-183.) part of the molecule is the major target for both the self-reactive (pathological) and innate like nAAbs. We did not find homology in the primary structures between the isolated huCS 145-150 peptide fragment and the nucleosome proteins; however, the isolated sequence motif (AALPSH) is hydrophobic and it is probably able to induce similar immunoreactivity as the also strongly hydrophobic nucleosome sequences. Our data call the attention for the general changes in self-recognition network under pathological conditions, which may result in cross-reactive epitope patterns.

Theoretical conclusions and future perspectives

In conclusion, we have successfully employed a random peptide library displayed on filamentous phage for the epitope mapping of two of our mAbs (anti-CD45RC and anti-HBX). We demonstrated the presence of CS recognizing nAAbs. We constructed a CS antigen fragment library displayed on phage lambda and showed, that the fine epitope pattern on CS is different under physiological and pathological (SLE) conditions. We identified cross -reactive epitopes on human and bacterial CS. In addition, we demonstrated cross reactivity of CS affinity-purified sera with nucleosome antigen. These data indicate that, in theory, nAAbs „specific" for a given self antigen could fulfill the function of participating in innate defense mechanisms and, at the same time, recognize a target antigen in a systemic autoimmune disease. On the basis of the limited immunoglobulin gene repertoire used for the production of nAbs, of the near germline sequence of these genes, and on the basis of the promiscuity in ligand binding of nAbs demonstrated in our present work, we speculate that nAbs in terms of their antigen recognizing characteristics are resembling the pattern recognition receptors mentioned in the introduction. Thus, at the level of recognized epitopes there is a possible new link between the innate like part and the adaptive-autoimmune arm of the humoral immune system. We summarized some features of nAAb mediated recognition substantiated by our results in Figure 9.

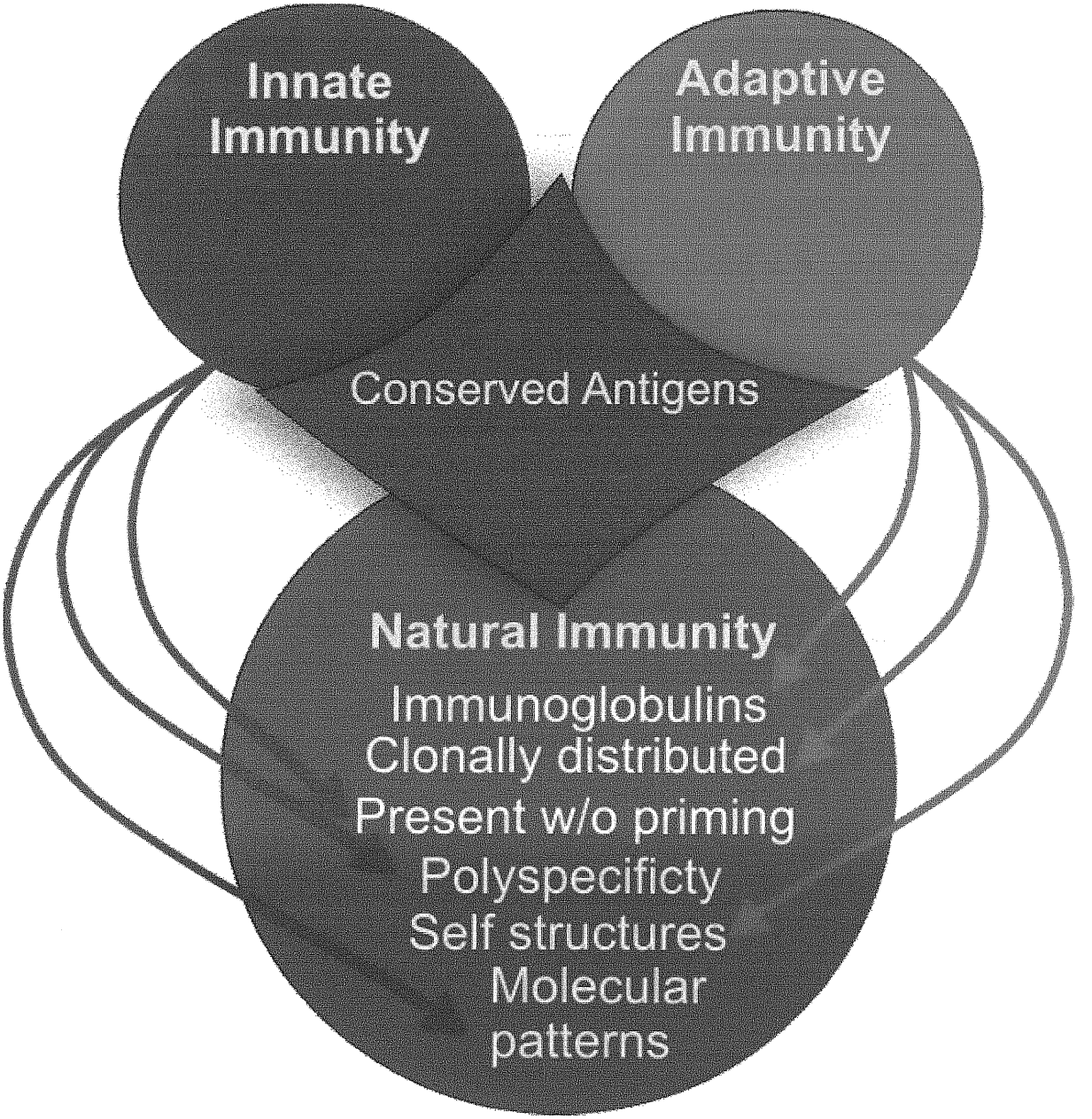


Figure 9. Characteristics of natural immunity mediated recognition.

Genetically conserved structures play a special role in various biological regulations, including metabolism, endocrine regulation, cell-cell interactions, intracellular signaling pathways and the immune response. Primary structure homologies between the antigens targeted in some autoimmune diseases and conserved sequences of different pathogens (viruses and bacteria) are well known. This so called "molecular mimicry" has been extensively studied however, direct causality of infections in the development of autoimmune diseases has only been verified in a few patients. These studies are more focused for homologies in primary structure, but, - as our results suggest - 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. Despite the fact that *E.coli* CS and mammalian CS exhibit long homologies in their primary structure, the cross-reactive epitopes which we identified by autoantibodies carry altered primary

structures with strong identical motifs in electric charge and hydrophobicity. Moreover, no homology could be found between the primary structure of nucleosome antigen and the sequence of mammalian CS identified as the cross reactive epitope. However, stretches of amino acids with a possibly similar physico-chemical molecular surface, are likely to be present, and the CS-specific IgM autoantibodies could recognize both antigens with a similar avidity. Our results are harmonizing with recently published data (Westal, 2005) which suggest a pivotal role of three-dimensional shape of conserved antigens in both targeting type immunity and tolerance.

Similarly to the initiation of immune response the maintenance of tolerance involves all three compartments of the immune system. Disturbances in co-operation among innate, natural, and adaptive immune system components may result in the impairment of both targeting type immune response and the self tolerance, thus paving the way for development of immunodeficiencies and pathological autoimmune phenomena.

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List of Tamás Czömpöly's publications

Publications related to this thesis:

Czompoly T, Olasz K, Simon D, Nyarady Z, Palinkas L, Czirjak L, Berki T, Nemeth P.
A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network?
Mol Immunol. 2006 Apr;43(11):1761-8 Impact factor: 3.19

Tamás Czömpöly, Árpád Lábadi, Mercédesz Balázs, Péter Németh, Péter Balogh
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APPENDIX

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A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network?

Tamás Czömpöly [a], Katalin Olasz [a], Diána Simon [b], Zoltán Nyárády [a,c], László Pálinkás [a], László Czirják [b], Tímea Berki [a], Péter Németh [a,*]

[a] *Department of Immunology and Biotechnology, Faculty of Medicine, University of Pécs, Szigeti út 12., Pécs H-7643, Hungary*
[b] *Department of Immunology and Rheumatology, Hungarian Brothers of St. John of God and University of Pécs, Irgalmasok u. 1., Pécs H-7621, Hungary*
[c] *Department of Oral and Maxillofacial Surgery, University of Pécs, Dischka Gy. u. 5., H-7621 Pécs, Hungary*

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Abstract

Natural antibody (nAb) producing B-1 B cells are considered an intermediate stage of evolution between innate and adaptive immunity. nAbs are immunoglobulins that are produced without antigen priming. nAbs can recognize foreign targets and may serve in the first line of immune defense during an infection. Natural autoantibodies (nAAbs) present in the serum of both healthy humans and patients suffering from systemic autoimmune diseases recognize a set of evolutionarily conserved self-structures. Because of their endosymbiotic evolutionary origin, proteins compartmentalized into mitochondria represent an interesting transition from prokaryotic foreign (non-self) to essential (self) molecules. We investigated the possible overlap in recognized epitopes of innate and self-reactive nAbs and surveyed changes in physiological autoreactivity under pathological autoimmune conditions. Epitope mapping analysis of a mitochondrial inner membrane enzyme, citrate synthase (CS) (EC 2.3.3.1) by synthetic overlapping peptides and phage display libraries using sera from healthy individuals and from patients having systemic autoimmune disease revealed CS recognizing nAAbs with IgM isotype. We analyzed cross reactive epitopes on human CS, bacterial CS, and various standard autoantigens. The anti-CS nAAbs by participating in the nAb network, could function in innate defense mechanisms and at the same time recognize a target antigen (nucleosome) in a systemic autoimmune disease. Thus, at the level of recognized epitopes there is a possible new link between the innate like component and the adaptive-autoimmune arm of the humoral immune system.

Keywords: Natural antibody; Mitochondrial inner membrane enzyme; Epitope mapping; Phage display; Multi-pin ELISA; Systemic lupus erythematosus; Autoimmunity

1. Introduction

Several lines of evidence indicate that natural antibody producing B-1 B cells represent an intermediate stage of evolution between innate and adaptive immunity. In fact they play an important role during the early phases of immune responses (Martin et al., 2001; Wardemann et al., 2002; Baumgarth et al., 1999). While they need to be under control and regulation because of their autoreactivity, these innate B cells themselves play an important role in controlling autoimmunity (Boes et al., 2000; Cocca et al., 2001).

Natural antibodies (nAbs) are immunoglobulins that are produced without immunization with antigen (Coutinho et al., 1995). These molecules can recognize foreign targets and may serve in the first line of immune defense during an infection (Ochsenbein et al., 1999). In contrast, natural autoantibodies (nAAbs) present in the serum of both healthy humans and patients suffering from systemic autoimmune diseases recognize a set of self-structures that have been conserved during evolution (Cohen and Young, 1991). Most nAAbs belong to the IgM or IgG isotype (Mouthon et al., 1995; Avrameas, 1991) and show polyreactivity with a broad range of affinities for the

* Corresponding author. Tel.: +36 72 536290; fax: +36 72 536289.
E-mail address: peter.nemeth@aok.pte.hu (P. Németh).

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recognized epitopes (Lacroix-Desmazes et al., 1998). Several functions have been suggested for nAAbs: they may participate in the selection of immune repertoires, play a role in the acceleration of primary immune responses (Ehrenstein et al., 1998), aid the clearance of apoptotic cells (Peng et al., 2005), possess anti-inflammatory effects (Miletic et al., 1996) and contribute to the maintenance of immune homeostasis (Lacroix-Desmazes et al., 1996).

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 a limited number of epitope mapping analyses performed on human antigens recognized by nAAbs. In particular little is known about the possible overlap in recognized epitopes of innate and self-reactive nAbs. Moreover, the possible changes in physiological autoreactivity under pathological autoimmune conditions remain unclear.

To address these problems we have chosen a mitochondrial inner membrane enzyme, citrate synthase (CS) (EC 2.3.3.1) for epitope mapping using sera of healthy individuals and patients having systemic autoimmune disease (systemic lupus erythematosus, rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyosits, systemic sclerosis, Raynaud syndrome and Sjörgen syndrome). We investigated the presence of CS recognizing nAAbs both in healthy and autoimmune patients. The molecular recognition pattern on CS was analyzed and compared under physiological and pathological (autoimmune) conditions. We analyzed cross reactive epitopes on human CS, bacterial CS, and various standard autoantigens. Our data indicate that the anti-CS nAAbs have an individually characteristic fine epitope pattern which is independent from the actual condition of the immune system by participating in the nAb network.

2. Materials and methods

2.1. Patients and control sera

Serum samples from healthy individuals: 63 Hungarian blood donors from the Blood Transfusion Service of Baranya county, Pécs (age: 36.8 ± 17.1 years; 32 women, 31 men); a standardized panel from 51 British blood donors and 176 Finnish blood donors (by the courtesy of professor G. Füst and Z. Prohaszka, 3rd Department of Internal Medicine at the Semmelweis University, Budapest); 44 serum samples from healthy infants from the Pediatrics Clinic, University of Pécs (age: 11.5 ± 4.5 months; 26 females, 18 males) and samples of patients with systemic autoimmune diseases: 326 clinically well-documented cases of systemic lupus erythematosus (SLE), rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyosits, systemic sclerosis, Raynaud syndrome and Sjörgen syndrome from the Immunology and Rheumatology Clinic, University of Pécs (age: 44.2 ± 14.1 years; 284 women, 42 men) were used in this work with the permit of the Ethical Committee of the Medical Center of the University of Pécs.

2.2. Detection of mitochondrial enzyme specific autoantibodies by ELISA

Ninety-six well polystyrene plates (NUNC) were coated with CS, malate dehydrogenase (MDH; EC 1.1.1.37) and pyruvate dehydrogenase (PDH; EC 1.2.4.1) from porcine heart (Sigma) in 0.1 M bicarbonate buffer, pH 9.6. Following the saturation of non-specific binding sites with 0.5% gelatin (Sigma) in PBS (pH 7.3), serum samples were incubated in triplicates at 1:100 dilutions in washing buffer (PBS, 0.05% Tween 20) for 60 min. Finally, the plate was incubated with HRPO conjugated anti-human-IgA, or -IgG or -IgM specific secondary antibody (Dako) for 60 min. The reaction was developed with *o*-phenylenediamine (Sigma), and measured on an iEMS MF microphotometer (ThermoLabsystem) at 492 nm. Cut-off values of each groups examined were calculated from the average of measured OD492 data. Sera having higher O.D. value than average + 2SD were considered positive. All measurements were standardized with a monoclonal anti-citrate synthase antibody (Clone 4H3-E5) we produced previously (Nemeth et al., 1991).

2.3. Affinity purification of sera on CS

CS from porcine heart was coupled to cyanogen-bromide activated sepharose 4B (Sigma) according to the manufacturer's instructions. Fifteen millilitres sera of 30 healthy blood donors and 14 patients with autoimmune disease were passed three times through the CS-sepharose resin. After washing antibodies were eluted in glycine-HCL pH 2.5, fractions were neutralized with 1 M Tris and were tested for CS reactivity with indirect ELISA using HRPO conjugated anti-human-IgA, or -IgG or -IgM specific secondary antibody (Dako).

2.4. Cross reactivity testing of CS affinity purified sera

Cross reactivity with additional mitochondrial inner membrane enzymes was tested with indirect ELISA using MDH and PDH from porcine heart (Sigma) as antigens.

Reactivity with *E. coli* CS was tested with pin-bound overlapping decapeptides as described previously (Petrohai et al., 2004; Nyarady et al., 2005).

Recognition of autoantigens implicated in various autoimmune diseases were tested using indirect ELISA kits developed for the quantitative measurement of double stranded DNA, nucleosome, Cenp-B, MPO, PR3, alpha-fodrin, gastric parietal cell, intrinsic factor, Asca, gliadin, tissue transglutaminase, cardiolipin, β2-glycoprotein-1, phosphatidyl serine, prothrombin (Orgentech) and Sm, RNP, SSA, SSB, Scl-70, Jo-1, CCP, thyreoglobulin, glomerular basal membrane (Hycor) specific autoantibodies.

2.5. Construction of a CS antigen fragment library

Total RNA was isolated using TriReagent (Sigma) from 3×10^6 mononuclear cells obtained by Ficoll Paque (Amer-

shamPharmacia) gradient centrifugation from peripheral blood of a healthy blood donor. Five micrograms of total RNA was reverse transcribed with Superscript II RT (Invitrogen) according to the manufacturer's instructions. cDNA encoding for the full length human mitochondrial citrate synthase was amplified with the following primers: 5′-ATGGCTTTACTTACTGCGGC-3′ and 5′-TTACCCTGACTTAGAGTCCAC-3′. The PCR reaction contained 300 mM of each dNTP, 1.5 mM $MgSO_4$, 1 μM of each primer, 5 μl cDNA and 5 units of ProofStart DNA polymerase (Qiagen) in a 100 μl final volume, cycling was done with the following profile: 95 °C 5 min, 35 cycles of 95 °C 1 min, 51 °C 30 s, 72 °C 2 min, final extension at 72 C for 10 min. The PCR product was separated on a 1.5% agarose gel and purified using the Quiaquick Gel Extraction Kit (Qiagen). Following A-addition it was cloned into a T/A vector using the InsT/Aclone PCR Product Cloning Kit (Fermentas). The identity of insert was verified by sequencing on an ABI3100 Avant genetic analyzer.

Library construction was done using the lambdaD-bio phage display vector (a kind gift from Dr. Alessandra Luzzago; Instituto di Ricerche di Biologia Molecolare, Italy) as described (Ansuini et al., 2002). In brief, inserts were produced by tagged random primed elongation and amplification using SpeI and NotI tagged random primers (Santi et al., 2000) and CS cDNA as template excised with BamHI and EcoRI (Promega) digestion from the plasmid mentioned above. Following purification with the Quiaquick PCR purification kit (Qiagen) and size selection on Wizard columns (Promega) inserts were digested with SpeI and NotI (Promega). Twenty ligations were set up containing 1 μg of SpeI/NotI digested lambdaD-bio DNA, 25 ng of SpeI/NotI digested insert, 30U of T4 DNA ligase (Fermentas) in a final volume of 5 μl and incubated 48 h at 4 °C. The ligation mixture was phenol-chloroform extracted, ethanol precipitated and packaged with the Ready To Go Lambda Packaging Kit (AmershamPharmacia). Phage were amplified by infecting log phase *E. coli* BB4 cells and plating them on LB agar plates. After plaque formation phage were eluted by an overnight incubation in SM buffer (100 mM NaCl, 8.1 mM $MgSO_4$, 50 mM Tris–HCl pH 7.5), concentrated with polyethylene glycol precipitation and resuspended in SM buffer supplemented with Complete EDTA Free Proteasae Inhibitor Cocktail (Roche).

2.6. Affinity selection of CS antigen fragment library

Affinity selection of CS antigen fragment library with CS affinity purified sera was performed essentially as described (Santini et al., 1998). Briefly, microtiter plates were coated with affinity purified anti-CS sera or anti-CS monoclonal antibody (mAb) 4H3E5 (developed in our lab) at 10 μg/ml in coating buffer. After blocking 10^{10} phage were incubated for 2 h at room temperature. Wells were washed five times and bound phages were recovered by in well infection of *E. coli* BB4 cells. The infected bacteria were plated on LB agar plates and phage were eluted then concentrated as described above. The affinity selection was repeated one more time and individual clones were picked up for DNA sequencing.

2.7. Random peptide library screening

The filamentous phage library displaying cyclic nine amino acid random peptides as a fusion to the N-terminal of the M13 major coat proteinVIII was constructed previously (Felici et al., 1991). Affinity selection of phages with CS affinity purified sera from SLE patients was performed using the biopanning technique (Parmley and Smith, 1988). In brief, microtiter plates were coated with affinity purified anti-CS sera (40 μg/ml during the first and 4 μg/ml during the second and third rounds of panning). After washing with PBS/0.05%Tween-20 and blocking with a solution containing PBS/3%BSA, 10^{10} ampicillin transducing unit (ATU) phage (blocked with PBS/1%BSA) was added and incubated for 2 h at room temperature. The plate was washed with PBS/Tween-20 (0.05% in the first or 0.5% in the second an third round of panning) and the bound phage were eluted with 1 mg/ml BSA/0.1 M glycine pH 2.2. Following neutralization with 2 M Tris, 10 ml XL1-Blue ($O.D._{600}$:0.5) was infected and plated on LB agar plates containing 50 μg/ml ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with 10^{11} plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37 °C, phage were precipitated with 16.7% PEG8000/3.3 M NaCl twice and resuspended in TBS. Enrichment was monitored by indirect ELISA with phage pools after each selection step. Following the third round of panning, randomly chosen clones were picked up and tested for reactivity with the selecting sera by indirect ELISA. Based on the ELISA results forty clones were selected for DNA sequencing.

3. Results

3.1. Anti-mitochondrial enzyme specific antibodies in healthy individuals and systemic autoimmune patients

Using simple binding ELISA we demonstrated the presence of antibodies recognizing CS, MDH, and PDC both in the sera of healthy individuals and systemic autoimmune patients. Isotype specific ELISA showed that enzyme specific antibodies with IgM isotype are more frequently present in all investigated groups than that of IgG or IgA isotypes (Table 1). No differences were found among the subgroups of healthy individuals; however, the incidence of anti-CS and anti-MDH autoantibodies with IgM isotype was significantly higher in autoimmune patients compared to the healthy controls.

We continued our investigations with CS specific IgM autoantibodies because this group showed the most characteristic pattern of distribution. We followed the titer of anti-CS IgM antibodies in 53 healthy individuals selected from British and Hungarian blood donors with repeated sample collection minimum three times during a 5-year period. We have found that the CS reactivity of individual sera remained permanently constant over this time period (Fig. 1).

Table 1
Prevalence of mitochondrial inner membrane enzyme specific antibodies in human sera

	CS IgA (%)	CS IgG (%)	CS IgM (%)	MDH IgA (%)	MDH IgG (%)	MDH IgM (%)	PDC IgA (%)	PDC IgG (%)	PDC IgM (%)
Hungarian blood-donors (63)	4	3	10	3	2	8	3	3	10
British blood-donors (51)	5	4	12	3	3	9	3	4	10
Finnish blood-donors (176)	5	3	9	2	2	8	3	5	10
Healthy infants (44)	2	2	7	4	4	9	2	4	9
Systemic autoimmune patients (326)	5	3	24	4	4	16	4	4	6

Sera were tested for citrate synthase (CS), malate dehydrogenase (MDH) and pyruvate dehydrogenase (PDH) reactivity with isotype specific indirect ELISA. Values in parentheses indicate the number of individual serum samples tested.

3.2. *Affinity purification and cross reactivity testing of CS reactive sera*

To exclude the masking effects of nonspecific bindings we purified anti-CS antibodies from 44 human sera (30 healthy and 14 autoimmune patients: 9 with SLE, 3 with systemic sclerosis and 2 with rheumatoid arthritis) by affinity chromatography for further experiments. Affinity purification was successful only in those cases (2 healthy and 2 SLE patients) when the actual serum had extraordinary high (OD492 > 1.5) anti-CS reactivity. The eluted anti-CS antibodies were exclusively with IgM isotypes (data not shown).

Cross reactivity of affinity purified anti-CS antibodies with other mitochondrial inner membrane enzymes (MDH and PDC) was tested by indirect ELISA. The affinity purified anti-CS antibodies have not recognized these enzymes (data not shown).

Subsequently, we applied a synthetic peptide fragment library in multi-pin ELISA to test whether antibodies affinity purified on CS from porcine heart (identity with human CS: 96%) would be able to recognize epitopes on bacterial CS from *E. coli* (identity with human CS: 25%). Only three cross reacting sequences were found: amino acids 124–133: FRRDSHPMAV (identity with human CS: 40%, similarity: 60%); amino acids 174–183: MCYKYSIGQP (identity with human CS: 30%, similarity: 40%) and amino acids 351–360: YFIEKKLYPN (identity with human CS: 40%, similarity: 60%).

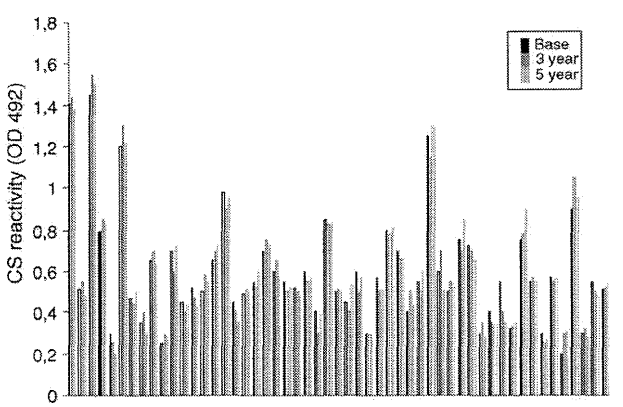

Fig. 1. CS reactivity in healthy individuals over a 5-year period. CS reactivity of sera from 53 healthy individuals was followed up during a 5-year period with IgM isotype specific indirect ELISA.

In order to examine the cross reactivity of affinity purified anti-CS sera on autoantigens having a role in various autoimmune diseases, we performed several indirect ELISAs with commercially available autoantibody kits. The affinity purified anti-CS sera from two SLE patients recognized nucleosome antigen (Fig. 2A). To exclude the possibility of CS contamination in the nucleosome antigen preparation we used our anti-citrate synthase mAb (Nemeth et al., 1991) in the same ELISA system. For further verification of our results we carried out competition ELISA experiments using CS from porcine heart as competitor (Fig. 2B).

In order to obtain further support for these findings we screened 46 additional SLE patients for CS and nucleosome reactivity and performed CS affinity purification from the 11 double positive (high CS and nucleosome reactivity) patients' sera. All of the above mentioned 11 CS affinity purified sera recognized the nucleosome antigen, which reactivity could be inhibited in competition with CS (mean nucleosome reactivity O.D. (450): 0.38 ± 0.15 without competition; 0.21 ± 0.09 in competition with 10 μg CS and 0.08 ± 0.03 in competition with 50 μg CS). In addition we performed fluorescent immunocytochemistry on Hep-2 cells using a commercially available kit from Diasorin. All of the CS affinity purified SLE patients' sera resulted a low-intermediate staining intensity in the nucleus – and the nucleoli of the nuclei were consequently negative – characteristic for the recognition of nucleosomes. We found an intermediate-high staining intensity in the cytoplasm, characteristic for the recognition of CS (data not shown). Our results show that there is indeed a cross reactivity of anti-CS antibodies from SLE patients with nucleosome antigen.

3.3. *CS antigen fragment library construction*

For analysis of fine epitope structure of anti-CS autoantibodies we constructed a CS antigen fragment library displayed on bacteriophage lambda. The library contains approximately 10^7 insert bearing independent clones. First we tested the library by performing an affinity selection with an anti-CS mAb. After the

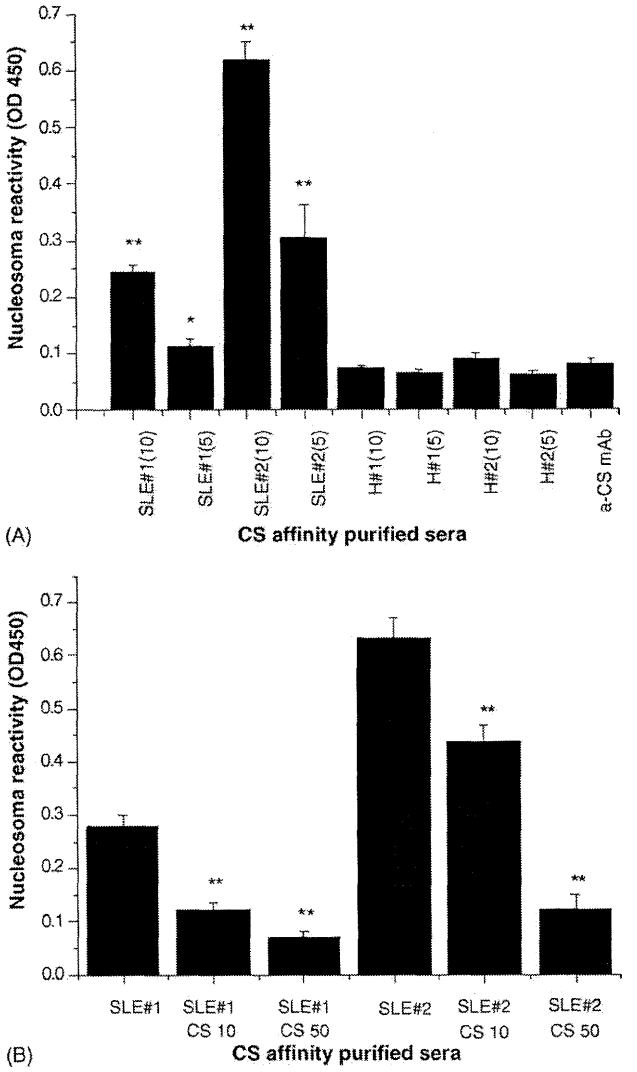

Fig. 2. Anti-CS antibodies from SLE patients recognize nucleosoma antigen. (A) Affinity purified anti-CS sera from two SLE patients (SLE) and two healthy (H) individuals were tested for nucleosome reactivity. Numbers in parentheses indicate the protein concentration in μg/ml. CS contamination of the nuclesome antigen was controlled with an anti-CS mAb 4H3E5 (a-CS mAb). (B) Competitive ELISA with CS: Affinity purified anti-CS sera from two SLE patients (10 μg/ml) were preincubated with the indicated amount of CS (μg/100 μl) for 1 h then reactivity for nucleosome antigen was tested. All measurements were performed in triplicates and were repeated in three independent experiments. $^{*}p<0.05$, $^{**}p<0.001$.

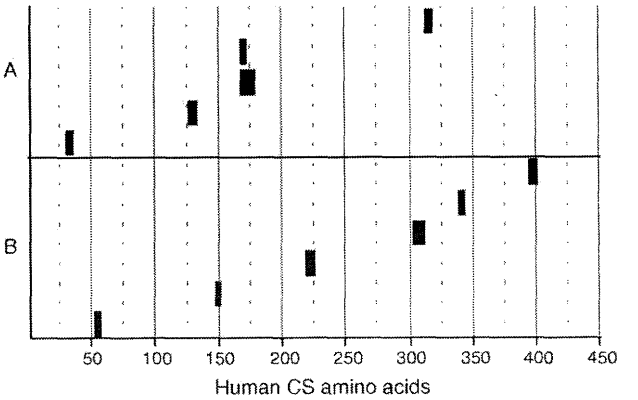

Fig. 3. Alignment of anti-CS sera selected phage clones with human CS. Deduced amino acid sequences of phage clones selected with affinity purified anti-CS sera prepared from healthy individuals (A) and patients with SLE (B) are plotted along the human CS sequence.

second round of affinity selection 30 clones were chosen for DNA sequencing. Among them four distinct sequences were found repeatedly (Table 2). These sequences could be aligned with amino acids 7–65 of human CS and the minimal epitope of our anti-CS mAb could be restricted to amino acids 31–59.

3.4. *Affinity selection of CS antigen fragment library with CS purified sera*

After the effectiveness of our phage displayed CS antigen fragment library for epitope mapping was demonstrated, we moved forward to the epitope mapping of affinity purified anti-CS sera. Following two rounds of affinity selection 20 clones selected with each serum were picked up for DNA sequencing. In contrast to the selection with our anti-CS mAb these clones carry short peptide sequences which could be also aligned to human CS (Fig. 3). These short sequences are scattered through the human CS sequence and it seems that practically the same regions of the molecule are recognized by the two groups of sera. However, it is important to note that the fine epitope pattern is different in the two groups examined.

3.5. *Random peptide library screening with CS purified sera*

To get additional data about the basis of CS-nucleosome cross reactivity we screened a nine amino acid random peptide library with CS affinity purified sera from two patients with SLE. The

Table 2
Alignment of anti-CS mAb selected phage clones with human CS

Number of independent isolations	Deduced amino acid sequence
5	AARLLGTKNASCLVLAARHASASSTNLKDILADLIPKEQARIKTFRQQHGKTVVGQITV
8	TNLKDILADLIPKEQARIKTFRQQHGKTV
9	ASCLVLAARHASASSTNLKDILADLIPKEQARIKTFRQQHGKTVVG
6	ASCLVLAARHASASSTNLKDILADLIPKEQARIKTFRQQHGKTV
huCS	AARLLGTKNASCLVLAARHASASSTNLKDILADLIPKEQARIKTFRQQHGKTVVGQITV

The number of independent isolations refers to the clones sequenced and found identical for the four distinct inserts. huCS indicates amino acids 7–65 of human CS.

Table 3
Alignment of CS affinity purified sera selected random peptides with human CS

Number of independent isolations	Deduced amino acid sequence
2	APASPYQCG
1	APASPYQSG
5	EAASPYQSH
5	YAAPSSQSH
9	YAAPSRQSH
18	YAAPSHQSH
huCS 145–150	AALPSH

The number of independent isolations refers to the clones sequenced and found identical for the given peptide. huCS indicates amino acids 145–150 of human CS.

40 sequenced clones carry similar peptides which show partial homology with human CS (Table 3) We performed competition ELISA with the phage clone most resembling CS (YAAPSHQSH, phage#5) both for CS and nucleosome antigen (Fig. 4). According to our results, phage#5 inhibited the CS reactivity of CS affinity purified sera from SLE patients but not from healthy individuals. When tested for blocking nucleosome reactivity, phage#5 proved to be an efficient inhibitor of the nucleosome reactivity measured with CS affinity purified sera from SLE patients. These results indicate that the CS-nucleosome cross reactivity at least in part is caused by antibodies recognizing the CS epitope (amino acids 145–150) mimicked by phage#5.

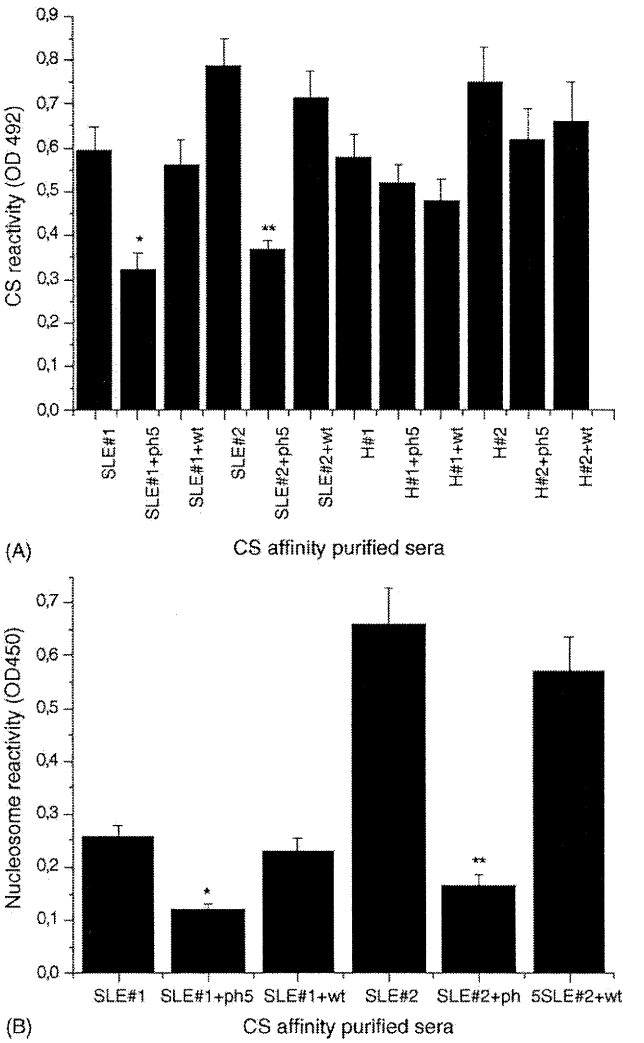

Fig. 4. Competition for CS and nucleosome antigen with a phage clone selected from a random peptide library. We performed competition ELISA with phage#5 (YAAPSHQSH), selected with CS affinity purified SLE patients' sera from a random peptide library. (A) CS affinity purified sera from patients with SLE or healthy individuals (SLE#1, #2 and H#1, #2 at 1 μg/ml) were preincubated with 10^{10} phage#5 (ph5) or wild type (wt) particles for 1 h at 4 °C, then reactivity for CS was measured by indirect ELISA. (B) CS affinity purified sera from patients with SLE (SLE#1, and #2 at 10 μg/ml) were preincubated with 10^{10} phage#5 (ph5) or wild type (wt) particles for 1 h at 4 °C, then reactivity for nucleosome antigen was measured by indirect ELISA. $^{*}p<0.05$, $^{**}p<0.005$.

4. Discussion

In this study we demonstrated the presence of natural antibodies recognizing CS both in healthy individuals and in patients with systemic autoimmune disease. Our finding that the majority of these antibodies have IgM isotype, their presence in infants and the long term stability of serum titers in adults indicates that these specificities belong to the nAAb repertoire established early in postnatal life (Mouthon et al., 1995; Stahl et al., 2000).

Since CS has not been implicated as target antigen in any autoimmune disease we investigated, it seems to be an appropriate model protein to examine potential changes in physiological autoreactivity under conditions of systemic autoimmune disease. In our previous study (Petrohai et al., 2004) we performed comparative epitope mapping on human CS with sera from healthy individuals and heart-transplanted patients using overlapping synthetic peptides. We detected similar recognition patterns in healthy group as in the present study, but in heart-transplanted patients both epitope pattern and the isotype of the anti-CS autoantibodies were different: strong dominance of IgGs was found with altered fine epitope specificities in strong relationship with the clinical stage (graft rejection).

Epitope mapping with overlapping synthetic peptides is a widely used technique (Maeji et al., 1995; Uray et al., 2003), but it's constraints include the in silico B-cell epitope prediction used for selection of antigenic regions (Nyarady et al., 2005), 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 performed the epitope mapping using a basically different technique: bacteriophage surface display of peptides is an extensively used technique for a variety of applications (Scott and Smith, 1990; McCafferty et al., 1990; Marks et al., 1991; Farilla et al., 2002). 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. 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. According to our results obtained with phage displayed antigen fragments, while

there is no favored region of the CS molecule recognized exclusively either by healthy individuals or patients with SLE, the fine epitope pattern is different in the two groups examined.

Since previous studies suggested that natural antibodies play an important role in the innate like component of the humoral immune response (Martin et al., 2001; Wardemann et al., 2002; Baumgarth et al., 1999), we investigated the possible overlap in nAAb recognized epitopes on mammalian and bacterial CS. Due to the prokaryotic origin of mitochondria, CS represents an attractive target molecule to examine the self-reactive nAAbs' capability to recognize epitopes on the foreign counterpart of the same molecule. To achieve this, we used sera affinity purified on mammalian CS for epitope mapping on CS from *E. coli*, using the overlapping synthetic peptide method. The three recognized sequence shows a limited homology with human CS, though identical amino acids with a possible anchor function are present at corresponding positions. These amino acids contain either polar or charged side chains, which is in agreement with previous reports about the preferential amino acid composition of nAAb epitopes (Novick et al., 1992; Pashov et al., 2002). The three peptides, according to the three-dimensional model, are located on the surface of the molecule. Moreover, two of the peptides (124–133 and 174–183), though separated by 50 amino acids in the primary sequence, are in close proximity on the structural model, indicating that they represent the same antigenic region.

We tested whether CS affinity purified nAAbs cross react with other self-antigens implicated in various autoimmune diseases. The surprising cross reactivity of SLE patients' CS affinity purified sera with nucleosome antigen could not have been unequivocally explained by the results of epitope mapping performed either by the synthetic overlapping peptide or phage displayed antigen fragment method. Therefore we screened a random peptide library displayed on filamentous phage. In this system random peptides are presented at high copy number, making the identification of low affinity interactions easier. We isolated a phage clone (phage#5) which carries a peptide corresponding to amino acids 145–150 of human CS, and while it has no effect on sera from healthy individuals, it is capable of inhibiting both the CS and nucleosome reactivity of CS affinity purified SLE patients' sera. Reviewing our data obtained by phage displayed CS antigen fragment library revealed that a lambda phage clone, carrying practically the same part of the CS molecule as the filamentous phage clone #5, was isolated with the SLE patients' sera. Therefore, on the basis of evidence substantiated by the random peptide and CS antigen fragment library methods, we conclude that this CS epitope (145–150) is preferentially recognized by the SLE patients' sera. The cross reactive epitope identified on human CS (145–150.) is located on the surface of the molecule. It is interesting to note that it is part of the region which contains two of the *E. coli* CS cross reactive determinants (124–133 and 174–183). We hypothesize that this (124–183) part of the molecule is the major target for both the self-reactive (pathological) and innate like nAAbs. We did not find homology in the primary structures between the isolated huCS 145–150 peptide fragment and the nucleosome proteins; however, the isolated sequence motif (AALPSH) is hydrophobic and it is able to induce similar immunoreactivity as the also strongly hydrophobic nucleosome sequences. Our data call the attention for the general changes in self-recognition network under pathological conditions resulting changes on cross-reacting epitope patterns.

In conclusion, we demonstrated the presence of CS recognizing nAAbs. We showed that, the fine epitope pattern on CS is different under physiological and pathological (SLE) conditions. We identified cross reactive epitopes on human and bacterial CS. In addition we demonstrated cross reactivity of CS affinity purified sera with nucleosome antigen. These data indicate that in theory, nAAbs "specific" for a given self-antigen could fulfill the function of participating in innate defense mechanisms and at the same time recognize a target antigen in a systemic autoimmune disease. Thus, at the level of recognized epitopes there is a possible new link between the innate like part and the adaptive-autoimmune arm of the humoral immune system.

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Use of cyclic peptide phage display library for the identification of a CD45RC epitope expressed on murine B cells and their precursors

Tamás Czömpöly, Árpád Lábadi, Mercedesz Balázs, Péter Németh, and Péter Balogh*

Department of Immunology and Biotechnology, Faculty of Medicine, University of Pécs, Szigeti út 12, Pécs H-7634, Hungary

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Abstract

The alternative splicing and variable expression of the exons near to the N-terminus of the leukocyte common antigen (L-CA, CD45) result in distinct extracellular isoforms expressed by cells with different functional and developmental properties. Here we report the tissue reactivity pattern and epitope specificity of a novel rat monoclonal antibody (IBL-8) against a restricted epitope of mouse CD45. We found that this mAb reacts with an epitope displayed by B cells and their precursors (both in newborn spleen and adult bone marrow). Moreover, peripheral CD8-positive T cells were also recognised at an intermediate intensity, whereas the CD4 T cell subset was weakly reactive. The epitope of this mAb was determined with M13 filamentous phages that display cysteine constrained nonapeptides on their coat proteins. The isolated bacteriophages expressing the putative epitope showed an isoform-specific inhibition of the binding of exon-specific mAbs. Deduced amino acid sequence data of these phages indicate that the epitope recognised by the IBL-8 mAb lies at the 136–144 region of the mouse CD45 molecule within its C exon, with a TAFP consensus sequence at its centre.

Keywords: Mouse CD45; Isoform; Exon C; IBL-8; Epitope phage display

CD45, a transmembrane phosphotyrosine phosphatase, is amongst the most abundant glycoproteins displayed by leukocytes [1,2]. It consists of a transmembrane and cytoplasmic region exerting catalytic function, whose part has a considerable degree of amino acid sequence homology among various mammalian species, and an extracellular part with a rather complex molecular structure. Unlike the intracellular domains, various parts of the extracellular region are remarkably different between leukocytes of distinct developmental and functional features. This complexity of CD45 is primarily due to the alternative splicing of its three domains spanning exons 4–6, denoted as exons A, B, and C, respectively, resulting in a characteristic CD45 isoform display by various lymphoid and myeloid cell populations. Irrespective of the extracellular variability, the intracellular region retains the same PTPase activity in regulating signal transduction via either antigen receptors or integrins [3]. B cells express all three exons, whereas T cell subsets alternatively use the B and C exons, and their expression pattern may change upon antigenic or other stimulation [4]. Moreover, the CD45 isoform expression pattern is considerably different between various species [5].

In addition to the differences caused by the alternative exon usage, the extensive glycosylation of CD45 protein further expands its structural complexity. It has been established that the variable exons are rich in O-linked oligosaccharide moieties linked to serine (S) or threonine (T) amino acids of the protein backbone [6]. In addition to being involved in potential selective receptor–ligand interactions of CD45, monoclonal antibodies can also be directed against such glycosylation-dependent determinants within these exons [7].

In mice, the mature B cells express the high molecular weight isoform of CD45, termed B220, which contains all three variable exons. While the detection of exon A has extensively been utilised to identify and characterise these cells in various tissue and cell samples, the expression pattern of the exon C on B cells and their precursors has been less thoroughly studied. We

* Corresponding author. Fax: +36-72-536-289.
E-mail address: peter.balogh@aok.pte.hu (P. Balogh).

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addressed this issue using a novel rat monoclonal antibody produced against an exon C determinant of the mouse CD45.

Recently for the precise identification of antigenic determinant for monoclonal antibodies, phage display technology has been used [8,9]. The availability of constructs at various lengths and configurations (linear or constrained) combined with the high throughput has made this approach attractive. In this system, filamentous phages are used as vehicles for displaying peptides on their minor coat protein III or major coat protein VIII. Using the monoclonal antibody as affinity ligand, the phages are enriched during several rounds of purification. After purification and nucleotide sequencing, the peptide sequence of their peptide construct can be resolved.

Our aim was to define the expression pattern of CD45RC isoform in mature and immature mouse B cells by using our new rat monoclonal antibody IBL-8 against the exon C. Furthermore, we sought to identify the antigenic determinant for this monoclonal antibody within the exon C with peptide phage display technique. Our data indicate that the expression of CD45RC determinant is closely linked to the appearance of CD45RA on mouse B cell precursors. The isolation of distinct types of phages displaying the peptide epitope indicates that the particular determinant within the CD45RC isoform is independent from the glycosylation of the molecule.

Materials and methods

Mice. Inbred female BALB/c mice between the age of 8 and 12 weeks or newborn pups were obtained from the University's SPF Animal Facility. The adult mice were kept under conventional conditions with rodent chow and water ad libitum. The lymphoid organs were removed from mice killed with cervical dislocation, in accordance with the guidelines set out by the Committee on Animal Experimentation of the University of Pécs.

Hybridoma cell lines and monoclonal antibodies. The IBL-8 (rat IgG1/k) hybridoma was produced in our laboratory. Briefly, Wistar rats were immunised with pooled mouse splenic lymphoid and stromal elements as previously described, and their splenic lymphocytes were fused with Sp-2/0 Ag14 cells with polyethylene glycol (PEG) mw. 4000 [10,11]. Following HAT-selection, the hybridoma clones were tested for antibody production employing large-scale immunohistochemistry of pooled supernatants. After repeated limiting dilution to ensure monoclonality, the IBL-8 clone was picked and characterised. For the phenotypic analysis of the mouse lymphocytes, anti-CD45RA/B220 IgG (RA3-6B2, BD Pharmingen, CA), anti-Thy-1 (IBL-1 [12]), anti-CD4 (YTS191.1.2, Serotec, UK), and anti-CD8α (IBL-3/25) mAbs were used. For multiple colour labelling FITC-conjugated anti-CD43 (S7, BD Pharmingen) mAb was used in conjunction with PE-labelled anti-B220 mAb (RA3-6B2, BD Pharmingen). For blocking experiments anti-CD45RA/B220 (RA3-6B2 IgG or 14.8 hybridoma cell line obtained from the American Type Culture Collection, VA), anti-CD45RC (GL24 [13]), and anti-pan-CD45 (IBL-5/25 [14]) were employed. The rat antibodies were detected using FITC-conjugated MRC OX-12 mouse anti-rat κ-chain mAb. For multiple labelling the IBL-8 mAb was purified on a Protein G affinity column using FPLC and conjugated with *N*-hydroxy-sulfo-succinimidobiotin ester (Sigma–Aldrich Kft, Budapest). The biotinylated mAb was purified on Sepharose G-25 column and detected using streptavidin–phycoerythrin (BD Pharmingen).

Flow cytometry. The cells from various lymphoid organs were released between the frosted ends of two glass slides into chilled PBS containing 0.1% BSA and Na-azide. Bone marrow cells were flushed from the femoral shafts using a G22 needle. The cells were filtered through a cell strainer and collected by centrifugation at 800*g* for 10 min at 4 °C. The pellet was resuspended in cold PBS–BSA and adjusted to 5×10^6 cells/ml. From this cell suspension 50 μl was added to 200 μl of primary antibody solution (1 μg/ml anti-CD45RA/B220 mAb or undiluted hybridoma supernatant, respectively) against mouse surface markers and incubated for 20 min on ice with repeated vortexing. After washing, the unlabelled antibodies were detected with FITC-conjugated OX-12 mAb. The unsaturated binding sites of the secondary antibody were blocked with incubating the cells in 20% normal rat serum in PBS–BSA for 10 min. After rinsing, 100 μl biotinylated IBL-8 mAb at 1 μg/ml concentration was added to the cells and further incubated for 20 min. The biotinylated mAb was detected using streptavidin–phycoerythrin. Following the removal of excess fluorophore, the cells were fixed in 1% paraformaldehyde in PBS and analysed using a Becton–Dickinson FACSCalibur equipped with He-laser using the CellQuest software package. The three-colour labelling was carried out using a cocktail of FITC-conjugated anti-mouse CD43 IgG, biotinylated IBL-8, and PE-labelled anti-B220 mAbs in the presence of FcγRII-blocking 2.4G2 mAb (BD Pharmingen) followed by the addition of streptavidin–CyChrome (BD Pharmingen). Controls included normal unlabelled, FITC-conjugated or biotinylated rat IgG at 1 μg/ml. Data from 10,000 electronic events gated on the forward and side scatter were collected and analysed. The labelling procedure for all flow cytometric analyses was repeated in at least three independent experiments.

The isoform-specificity of the IBL-8 mAb was determined using a competition binding with reference mAbs, including anti-CD45RA/B220 (RA3-6B2) and anti-CD45RC (GL24) mAbs. After incubating the cells with these competitor antibodies at 10 μg/ml, the cells were reacted with biotinylated IBL-8 mAb, followed by washing. The biotinylated IBL-8 was detected with streptavidin–phycoerythrin. The degree of competition was determined by comparing the mean fluorescence intensity (MFI) to that of the maximal (normal rat IgG as competitor) and minimal (IBL-8 mAb as competitor) labelling.

The inhibitory effect of phages displaying the putative IBL-8 epitope was determined using phages purified with PEG precipitation (see below). The phages (10^{12}/ml) were incubated with 1 μg/ml purified mAb in the final volume of 100 μl for 30 min at room temperature, then to the phage–mAb mixture 50 μl lymph node cell suspension was added, prepared as above. After 20 min on ice, the cells were washed and incubated with phycoerythrin-conjugated goat anti-rat IgG (BD Pharmingen). The reaction was stopped by repeated washing, the cells were fixed, and the samples were analysed by flow cytometry. Controls included anti-CD45RA/B220 mAb in place of IBL-8 mAb and M13 phages replacing the IBL-8 epitope expressing phages.

Screening of the phage displayed random peptide library. The filamentous phage library displaying cyclic nine amino acid random peptides as a fusion to the N-terminal of the M13 major coat proteinVIII was constructed previously [15] and was a generous gift from Dr. Alessandra Luzzago (Instituto di Ricerche di Biologia Molecolare, Rome, Italy). The affinity selection of phages with IBL-8 mAb was performed using the biopanning technique [16]. In brief, a plastic petri dish was coated with the mAb overnight. After washing with PBS/0.05% Tween 20 and blocking with a solution containing PBS/3% BSA, 10^{10} ampicillin transducing unit (ATU) phage (blocked with PBS/1% BSA) was added and incubated for 2 h at 37 °C. The plate was washed with PBS/Tween 20 and the bound phages were eluted with 1 mg/ml BSA in 0.1 M glycine, pH 2.2. Following neutralisation with

2 M Tris–base, 10 ml *Escherichia coli* bacterial strain XL1-Blue (OD_{600}: 0.5) was infected and plated on LB agar plates containing 50 μg/ml ampicillin. The next day the colonies were scrapped off the plates, resuspended in 10 ml LB, and superinfected with 10^{11} plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37 °C phages were precipitated with 16.7% PEG8000/3.3 M NaCl twice and resuspended in TBS. Following the second round of panning 10 ml XL1-Blue (OD_{600}: 0.5) was infected with 10^4 ATU phage, superinfected with 10^{11} PFU M13KO7 helper phage, and plated on LB agar plates at a low density. After 5 h of incubation, nitrocellulose membranes were applied and left on the plates overnight. The membranes were blocked with TBS/0.05% Tween 20/5% non-fat dried milk and incubated with the IBL-8 mAb (1 μg/ml in blocking solution) for 2 h. Positive clones were visualised with a HRPO-conjugated anti-rat IgG secondary antibody (BD Pharmingen, CA) using 9-amino-ethyl carbazol as chromogen in a 0.1 M Na–acetate buffer (pH 5.2) and 0.1% H_2O_2.

Phage ELISA. ELISA plates (NUNC, USA) were coated with 10^{10} phage particles overnight at 4 °C in triplicate samples. After blocking with TBS/2% non-fat dried milk, the wells were incubated with the IBL-8 mAb (2 μg/ml in TBS/1% non-fat dried milk) for 2 h. Reactions were developed with a HRPO-conjugated anti-rat IgG secondary antibody using *ortho*-phenylene diamine as chromogene in a 0.1 M Na–citrate buffer (pH 5.2) and 0.01% H_2O_2, and photometrically quantitated at 492 nm using a Dynatech MR200 ELISA reader.

Sequencing. The DNA of the selected phage clones was isolated by phenol extraction of phages precipitated with PEG and sequenced with BigDye 1.0 chemistry on an ABI Prism 310 instrument (Applied Biosystems, USA) using M13-20 primer.

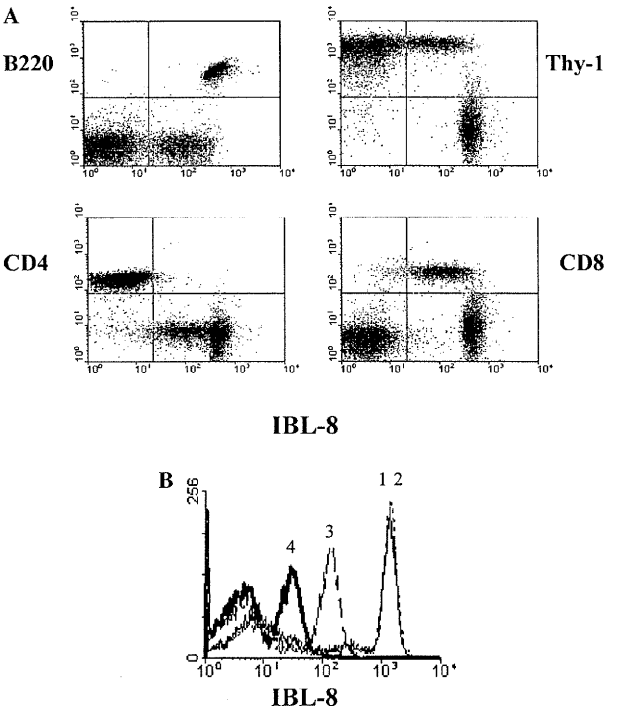

Fig. 1. Tissue reactivity and relative isoform-specificity of IBL-8 mAb. (A) Mouse lymph node cells were labelled with antibodies specific for mouse lymphocyte subset markers (indicated at the upper right or left corner of each panel) and IBL-8 mAb. The plots are representative of three independent experiments. (B) The effect of preincubation of target cells with CD45 isoform-specific mAbs on the binding of IBL-8 IgG. The numbers at the individual curves denote the following competitor reagents: 1, irrelevant IgG; 2, anti-CD45RA mAb RA3-6B2; 3, CD45RC-specific mAb GL24; and 4, IBL-8 IgG (4). Numbers at the *y*-axis indicate the number of events.

Results

Relative epitope specificity of IBL-8 mAb within the exon C product of mouse CD45

Our preliminary immunohistochemical and flow cytometric data indicated that the antigen recognised by the IBL-8 rat mAb was primarily expressed by B cells, probably related to a B-cell specific isoform of mouse CD45. In order to determine the expression pattern of IBL-8 antigen, a flow cytometric analysis was carried out employing antibodies against murine lineage-specific markers B220, Thy-1, CD4 and CD8, and the IBL-8 mAb. We found that the B cells isolated from a variety of peripheral lymphoid tissues (spleen, lymph nodes, and Peyer's patches) were homogeneously and intensely reactive with this mAb, while the T cells had a rather heterogeneous staining. Most of the $CD4^+$ T cells were negative or dim, whereas the majority of $CD8^+$ T cells had an intermediate level of reactivity between those of $CD4^+$ T cell subset and B cells, respectively (Fig. 1). This distribution is characteristic for the expression pattern of murine CD45RC isoform [13]. The mutually proportional display of the putative CD45 epitope recognised by the IBL-8 mAb and the exon A product identified by anti-CD45RA/B220 mAb indicates a close sterical relationship within the variable region of CD45 molecule (upper left panel in Fig. 1A), manifested in the diagonal arrangement of double positive B cell population in the dot plot. A simultaneous staining with a pan-CD45 specific mAb (IBL-5/25) revealed that, under the same labelling conditions, the overwhelming majority of CD45 molecules expressed on B cells contain both the B220 and the IBL-8 epitope, whereas the $CD8^+$ T cells have a heterogeneous expression pattern. Estimated upon the mean fluorescence intensity of IBL-8 labelling relative to that of pan-CD45, approximately 80–90% of the total CD45 molecules displayed by individual $CD8^+$ T cells lack the IBL-8 epitope.

In order to define the relative epitope specificity of IBL-8 mAb amongst the various variable exons of the CD45 molecule, a competitive antibody-mediated binding inhibition assay was performed. We found that while the CD45RA-specific mAbs (RA3-6B2 and 14.8, respectively) did not have any inhibitory effect upon the subsequent binding of IBL-8 mAb to mouse target cells, the addition of the CD45RC-specific mAb GL24 almost completely abrogated it (Fig. 1B). The similarity between the tissue reactivities of GL24 and IBL-8 mAbs together with the efficient blocking of IBL-8 mAbs antigenic binding by preincubation with the CD45RC-specific

GL24 mAb strongly suggests that they are directed against the same variable region within the CD45 molecule.

Expression pattern of IBL-8 epitope of CD45RC during the primary B-cell differentiation

The display of exon A of the CD45 on B-lineage cells only has been exploited for the identification of these cells as well as their immature precursors in primary lymphohaemopoietic tissues [17]. We sought to determine the display of IBL-8 epitope by immature B-cell precursors and correlate it with that of the B220 determinant. CD43 antigen was chosen as a developmental marker, whose level of expression inversely correlates with the progress of B-cell maturation. Thus the less differentiated B cell precursors are characterised as $CD43^{hi}/B220^{+}$ cells, whereas the more mature cells acquire $CD43^{lo}/B220^{+}$ phenotype [18]. Application of a cocktail of FITC-conjugated anti-CD43, PE-labelled anti-B220, and biotinylated IBL-8 mAbs developed with streptavidin–CyChrome to lymphoid cells from newborn spleen or young adult bone marrow revealed that the maturation of B cells is accompanied with the simultaneous upregulation of both the CD45RA and RC determinants (Fig. 2). Thus an approximately 2–4-fold upregulation of IBL-8 marker expression could be detected during the transition from $B220^{lo}/CD43^{+}$ to $B220^{hi}/CD43^{-}$ phenotype. This regulated increase of expression was independent from the tissue source (newborn spleen or adult bone marrow) of B-cell precursors. As a result of parallel increase of both RA and RC epitopes, we could not identify any distinct B cell precursor population with a possible combination of intermediate (RA^{low}/RC^{hi} or RA^{hi}/RC^{lo}) CD45 phenotype. In addition, the myeloid cells (identified by their forward and side light scatter features) did not react with this antibody (not shown).

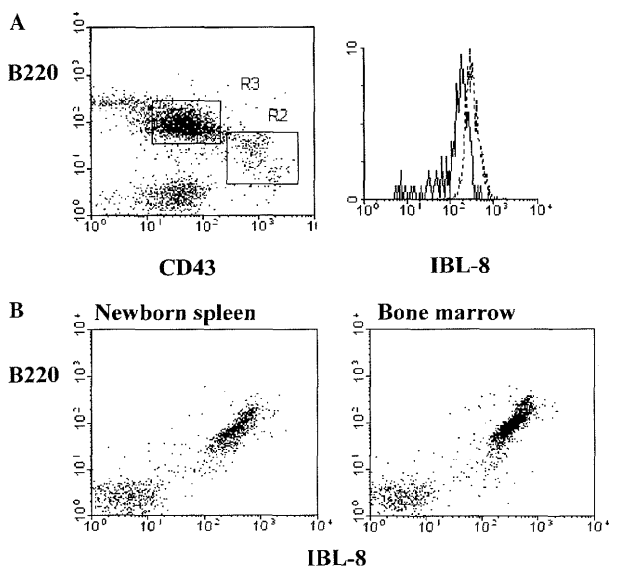

Fig. 2. Differential expression of CD45RC isoform by immature and mature B cell precursors. (A) Bone marrow mononuclear cells were resolved into immature ($CD43^{hi}/B220^{lo}$—R2 gate) and more differentiated ($CD43^{lo}/B220^{hi}$—R3 gate) subsets (dot-plot panel). The histogram shows the IBL-8 reactivity of these different subpopulations (R2, continuous line; R3, dashed line). (B) Simultaneous upregulation of CD45RA and CD45RC exon expression in developing B cell precursors from various haemopoietic sources.

Identification of IBL-8 epitope within the mouse CD45RC determinant with peptide phage display

In order to determine the epitope recognised by the IBL-8 mAb within the CD45RC determinant, we have screened a cyclic random peptide library displayed on M13 filamentous phage. On the basis of immunoscreening and ELISA test, we have chosen 20 clones for DNA sequencing. In addition to the serological tests of the selected clones, we also assayed the phages' capacity to interfere with the antibody binding to the native antigen displayed on mouse B cell surface using flow cytometry. Among these 20 clones we have identified two types of clones displaying slightly different nonapeptides. A comparison of the deduced amino acid sequence of these two groups of clones with the primary sequence of the CD45 molecule assigns the epitope recognised by the IBL-8 mAb to amino acids 136–144 (ADTAFPVDT) (Table 1). This sequence lies within the exon C of the mouse CD45 molecule. Preincubation with both cA3 and cB6 phages as prototypes for the two groups completely inhibited the subsequent recognition of native antigen by IBL-8 mAb (Fig. 3). It was interesting to note, however, that the same phages exerted only a reduced inhibitory effect for the binding of GL24 reference mAb to CD45RC, indicating that its epitope specificity is slightly different from that of IBL-8.

Discussion

The structure of CD45 antigen poses a remarkable duplicity in its assembly. The intracellular, enzymatically active region has preserved an extensive homology across phylogeny, whereas the variable exons within the extracellular region can be present in different numbers, combinations, and glycosylation variants between various lymphohaemopoietic cells, as dictated by their developmental and functional properties [1,2]. Here we describe the expression pattern of the most membrane-proximal variable exon of the extracellular region by mouse mature lymphoid cells, as well as on B cell precursors using IBL-8 rat monoclonal antibody. The technique of epitope phage display library [8,9] has been used to identify the antigenic determinant recognised by this new mAb.

Table 1
Alignment of the amino acid sequence of CD45RC exon to the IBL-8 phage epitopes

	Amino acids	Number of independent isolations
Mouse CD45 C domain	PGERTVPGTIPADTAFPVDTPSLARN	
cA3 phage clone	AYTAFPLD	13
cB6 phage clone	NTAFPSGTS	7

The amino acid sequence spanning the 125–150 region of murine CD45 as a reference and the nonapeptide sequences of peptides displayed are shown. The consensus amino acids between the region 136 and 144 are underlined. The numbers of independent isolates refer to the clones sequenced and are found identical for the two distinct groups.

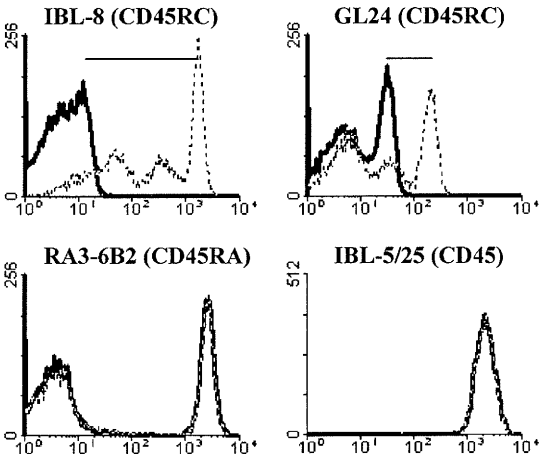

Fig. 3. Specific inhibitory effect of phages displaying the IBL-8 epitope. cA3 phages were reacted with various anti-CD45 monoclonal antibodies (indicated at the top of each plot) followed by adding the phage–mAb mixture to mouse lymph node cells. The mAb binding was determined using PE-conjugated anti-rat IgG. Bold curves represent the samples with cA3 phage, the bold curves illustrate the control labelling following preincubation with "empty" M13 phages. The horizontal lines in the anti-CD45RC mAb panels indicate the shift of peak channels of fluorescence.

Our finding indicates that, similar to the CD45RA isoform expression, among lymphoid cells the CD45RC expression is highest on B cells. The myeloid cells do not appear to express this isoform, similar to the lack of CD45RA isoform on these cells. The display of both CD45RA and CD45RC epitopes in quantitative flow cytometry appeared to be at the same fluorescence intensity as a pan-CD45 determinant under identical labelling conditions, which suggests that most, if not all, of the CD45 molecules produced by B cells contain both isoforms. The acquisition of high-level co-expression of CD45RA and CD45RC determinants takes place in cells already past the pre-B cells stage, as confirmed by the higher level display of CD45RC by $B220^{hi}/CD43^{-}$ cells. It was interesting to note that, despite the different ontogenic sources (neonatal splenic B cells formed from foetal vs. adult lymph node B cells from bone marrow-derived adult haemopoietic stem cells), the B cell precursors in the newborn spleen and bone marrow had the same CD45RA/RC phenotype, unlike the differences in their display of other differentiation antigens, including CD5, CD21, and CD43 [19].

While the B cells were found intensely labelled, the reactivity of CD8 positive T cells was significantly weaker for CD45RC and negative for CD45RA, as previously reported [13]. As a result, the CD45RC-containing fraction of the total CD45 pool produced by CD8 T cells was a small minority (approximately 10–20%) as assessed by the weaker fluorescence intensity with anti-CD45RC mAb related to an anti-pan-CD45 mAb. This discrepancy may be important in the different abilities for recognition of putative CD45RC ligands by these two lymphocyte subsets.

In order to define the epitope recognised by this anti-CD45RC mAb, we applied cysteine-constrained peptide epitope phage display technology. We could isolate two groups of phages, with largely overlapping peptide sequences that corresponded to that of mouse CD45RC exon. It appears that the consensus TAFP peptide needs to be continuous in the non-terminal region of this epitope, where the single- or double-residue alterations of the flanking amino acids still permit the effective recognition of the epitopes by IBL-8 mAb. It is also interesting that the threonine residue initiating this sequence does not need to be glycosylated for the efficient recognition by antibody in this heavily O-glycosylated variable region of the CD45 antigen, as the peptide epitope expressed in the absence of eukaryotic post-translational modifications could completely block the mAbs binding to the native structures. Moreover, this relatively small region within the extracellular part of CD45 molecule may potentially contain other antigenic determinants as well, as the IBL-8 reactive peptide epitopes could only partially block the antigen binding by the GL24 anti-CD45RC reference mAb. For further studies addressing the importance of this region of the CD45 in the lymphocyte development and function, both this novel CD45RC-specific mAb and the corresponding epitope peptides may prove useful.

Acknowledgments

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Determination of the fine epitope specificity of an anti-hepatitis B virus X protein monoclonal antibody using microanalytical and molecular biological methods

József Pál[a], Tamás Czömpöly[a], Zoltán Nyárády[b], Ilona Marczinovits[c], Tamás Janáky[d], Zoltán Kele[d], Franco Felici[e], Péter Németh[a,*]

[a] *Department of Immunology and Biotechnology, Faculty of Medicine, University of Pécs, Szigeti u. 12, Pécs H-7643, Hungary*
[b] *Department of Dentistry, Oral and Maxillofacial Surgery, University of Pécs, Pécs, Hungary*
[c] *Department of Physiology, Faculty of Medicine, University of Szeged, Szeged, Hungary*
[d] *Department of Chemistry, Faculty of Medicine, University of Szeged, Szeged, Hungary*
[e] *Department of Microbiological Genetic and Molecular Sciences, University of Messina, Messina, Italy*

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Abstract

The recombinant form of the 17 kDa, highly hydrophobic and disulfide-bonded hepatitis B virus X protein (HBX) was used for developing a set of monoclonal antibodies (Mab). Our present goal was to determine the fine epitope specificity of our anti-HBX Mab. Based on computer analysis two sequences (amino acids 22–31 and 100–114) were predicted for possessing high immunogenity while the anti-HBX Mab did not recognized them. Limited proteolysis and mass spectroscopic analysis suggested another possible sequence (amino acids 14–26), which also proved to be negative using an immunoserological test. Subsequently, we performed a screen of a phage displayed random peptide library, by which we could localize the epitope to amino acids 88–93. This finding was confirmed using three overlapping fusion peptides spanning amino acids 77–142. Their testing in ELISA assigned the epitope to amino acids 77–95, which supports the result obtained by screening the phage displayed library. Our results suggest the necessity of a complex application of current molecular biological and immunological techniques in fine structure mapping. This approach will be useful to study the prognostic relevance of different antigenic sites on HBX during the development of chronic hepatitis and primary hepatocellular carcinoma.

Keywords: Monoclonal antibody; Hepatitis B virus X protein; Epitope mapping; Mass spectrometry; Phage display

1. Introduction

Hepatitis B virus (HBV) is an important etiologic agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. (Szmuness, 1978; Tiollais et al., 1985; Beasley and Hwang, 1994; Feitelson and Duan, 1997). The smallest open reading frame of HBV called X, encodes a 17 kDa protein of 154 amino acid with highly hydrophobic and disulfide-bonded characters (Caselmann, 1996; Gupta et al., 1995). The X protein (HBX) is a multifunctional regulatory protein which modulates transcription, cell responses to genotoxic stress, protein degradation, and signaling pathways, but the precise function of HBX is not well understood (Andrisani and Barnabas, 1999; Murakami, 1999, 2001).

We developed a set of monoclonal antibodies (Mab) against the HBX for research and diagnostic use (Pal et al., 2001). The determination of fine epitope structure recognized by one of our Mab became necessary due to the results of our retrospective study on human liver biopsy specimens. The various epitopes of HBX antigen are attributed with different functional importance (Stemler et al., 1990; Murakami, 1999). Detection of their expression pattern seems to be a suitable prognostic factor in laboratory diagnosis (Feitelson et al., 1990; Vitvitski-Trepo et al., 1990; Kumar et al., 1998). Moreover, for the quantitative measurement of the HBX from human serum, we developed a sandwich type ELISA using a pair of anti-HBX Mabs with no overlapping epitopes. Our goal was to map the fine epitope-specificities of the antibodies that were used in the retrospective immunohistochemical study and in other immunoserological assays.

* Corresponding author.
E-mail address: peter.nemeth@aok.pte.hu (P. Németh).

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2. Materials and methods

2.1. Materials

Recombinant HBX (amino acids 1–154) was produced as a glutathione *S*-transferase (GST) fusion protein as described (Marczinovits et al., 1997).

Monoclonal antibodies against the recombinant HBX were developed earlier (Pal et al., 2001), the Mab used in this study was the product of clone 3F6-G10. The *E. coli* strain XL1-Blue MRF and the helper phage M13KO7 was purchased from Stratagene (USA). The *E. coli* strain DH5-alfa was from Invitorgen (USA). Nitrocellulose membrane was purchased from Pharmacia Biotech (Sweden). Alkaline phosphatase (AP) conjugated anti-mouse IgG antibody and horseradish-peroxidase (HRPO) conjugated anti-mouse IgG antibody were purchased from Dako (Denmark). Luria-Bertani (LB) bacterial media and all the other chemicals were purchased from Sigma (Germany).

2.2. Antigenity prediction and synthetic peptides

The Chou–Fasman and the Garnier–Osguthorpe–Robson secondary structure predictions (Chou and Fasman, 1974; Garnier et al., 1978; Gibrat et al., 1987; Prevelige and Fasman, 1990) of HBX were performed with the Protein Structure program of the Wisconsin-GCG package (Genetics Computer Group Inc., University Research Park, Madison, WI, USA) peptide sequences chosen on the basis of Jameson–Wolf antigenity index were synthesized using fmoc chemistry and purified with HPLC at the Department of Chemistry, Faculty of Medicine, University of Szeged.

2.3. Limited proteolysis and mass spectrometry

Recombinant X protein was reduced by dithiothreitol, alkylated with iodoacetamide and digested by trypsin in solution (Kele et al., 1998). The peptide fragments were separated on a C18 reversed-phase HPLC column (Nucleosil 5C18, 300 Å) using 40 min linear gradient from 20 to 60% solvent (0.1% trifluoroacetic acid in 80% aqueous acetonitrile) at the flow rate of 1 ml/min solvent. Column effluent was monitored at 215 nm and was collected in 1 ml fractions. Binding characteristic of the separated peptides to monoclonal antibody against HBX were tested by ELISA. The amino acid sequence of the fractions showing the highest positive reaction in ELISA was determined by nano-ES/MS/MS using a TSQ 7000 triple quad mass spectrometer (Wilm and Mann, 1996).

2.4. Screening of the phage displayed random peptide library

The filamentous phage library displaying nine amino acid random peptides as a fusion to the N-terminal of the M13 major coat protein VIII was constructed previously (Felici et al., 1991), and was a generous gift from Dr. Alessandra Luzzago (Instituto di Ricerche di Biologia Molecolare, Italy). The affinity selection of phages with our anti-HBX Mab was performed using the biopanning technique (Parmley and Smith, 1988). In brief, a plastic petri dish was coated with the Mab overnight (40 and 4 μg/ml in PBS during the first and the second round of panning, respectively), after washing with PBS/0.05% Tween-20 and blocking with a solution containing PBS/3% BSA, 10^{10} ampicillin transducing unit (ATU) blocked phage was added and incubated for 2 h at 37 °C. The plate was washed with PBS/Tween-20 (0.05% in the first or 0.5% in the second round of panning) and the bound phage were eluted with 1 mg/ml BSA/0.1 M glycine, pH 2.2. Following neutralization with 2 M Tris–base, 10 ml XL1-Blue ($O.D._{600}$: 0.5) was infected and plated on LB agar plates containing 50 μg/ml ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with 10^{11} plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37 °C phage were precipitated with 16.7% PEG8000/3.3 M NaCl twice and resuspended in TBS. Following the second round of panning 10 ml XL1-Blue ($O.D._{600}$: 0.5) was infected with 10^4 ATU phage, superinfected with 10^{11} PFU M13KO7 helper phage and plated on LB agar plates at a low density to allow immunoscreening to be performed. After 5 h of incubation nitrocellulose membranes were applied and left on the plates overnight. The membranes were blocked with TBS/0.05% Tween-20/5% non-fat dry milk and incubated with the anti-HBX Mab (1 μg/ml in blocking solution) for 2 h. Positive clones were visualized with an AP conjugated anti-mouse IgG secondary antibody.

2.5. Phage ELISA

For each clone three wells of an ELISA plate (NUNC, USA) were coated with 10^{10} phage particles overnight at 4 °C. After blocking with TBS/2% non-fat dry milk the wells were incubated with the anti-HBX Mab (2 μg/ml in TBS/1% non-fat dry milk). Reactions were developed with a HRPO conjugated anti-mouse IgG secondary antibody.

2.6. Sequencing

The DNA of the selected phage clones was isolated by phenol extraction and sequenced with BigDye 1.0 chemistry on an ABI Prism 310 instrument (Applied Biosystems, USA).

2.7. Construction of recombinant overlapping HBX fragments and production of bacterial fusion proteins

For the production of recombinant overlapping HBX fragments we used the glutathione S-transferase Gene

Table 1
PCR primers used for the generation of DNA encoding for fragments of HBX

Sequence of primers (5′–3′)	Peptide fragments, AA
GAGGATCCATGGAGACCACCGTGAAAGC CTGAATTCCCCAAACAGTCTTTGAAGTATGCC	77–116
GAGGATCCAGGACTCTTGGACTCTCTG CTGAATTCCCGCCTCCTAATACAAAGACCT	96–135
GAGGATCCTTTAAAGACTGGGAGGAGTTG CTGAATTCCCGGCAGAGGTGAAAAAGTTGC	116–142

Recognition sites of the restriction endonucleases are underlined (*Eco*RI: GAGGATCC; *Bam*HI: CTGAATTC). AA: corresponding amino acids of the HBX.

Fusion System (Amersham Pharmacia Biotech, Sweden). Three overlapping parts of the HBX gene (subtype ayw, Bichko et al., 1985) were amplified by PCR using primers with *Bam*HI and *Eco*RI restriction sites (Table 1). The fragments were cloned into the expression vector pGEX-6P-1. Each construct was verified by sequencing. Fusion proteins were expressed in *E. coli* DH5-alfa, and the recombinant proteins were purified using the GST–glutathione affinity system with modifications according to Marczinovits et al., 1997.

2.8. ELISA with overlapping fragments

Microtiter plates (Nunc, USA) were coated with 0.1 μg antigen in 0.1 M bicarbonate buffer, pH 9.6, at 4 °C overnight. PBS-gelatin was used to block the free binding sites. The anti-HBX Mab was used at final concentration of 1 μg/ml for 1 h at 37 °C. Reactions were developed by a HRPO labeled anti-mouse IgG secondary antibody.

3. Results

Computer analysis of the HBX secondary structure with the Chou–Fasman and the Garnier–Osguthorpe–Robson structure predictions revealed that there were a few sequences exhibiting turn structures along the N-terminal half of the molecule and at the C-terminal as well. The sequence between amino acids 75–130 contains alpha-helices. Beta-sheets are predicted all along the polypeptide chain. There are no sites presumed to be glycosylated. The protein contains 84 hydrophobic amino acids amounting to 54% of the whole molecule. On the basis of the Jameson–Wolf antigenity index, two sequences (amino acids 22–31 and 100–114) were chosen and synthesized for further testing (Fig. 1). The prediction was controlled immunoserologically with simple binding ELISA technique using synthetic peptides (sequences 22–31 and 100–114) as antigens. Unfortunately, none of the Mabs recognized these epitopes (data not shown).

Using our anti-HBX Mab we screened a random peptide library displayed on filamentous phage. Based on the immunoscreening and ELISA tests we chose 10 phage clones for DNA sequencing. A comparison of the deduced amino acid sequences of these clones revealed a consensus sequence of LPxxLH (Table 2). This sequence can be found in the primary structure of HBX (amino acids 88–93). This is in agreement with our earlier Western-blot result performed with recombinant HBX antigen under reducing conditions (Pal et al., 2001).

Limited proteolysis and mass spectroscopic analysis were performed to compare the results of computer prediction and phage display techniques. Limited proteolysis of the recombinant HBX was performed with trypsin digestion, and the peptides separated by reversed-phase HPLC were tested in ELISA. We found strong reactions with anti-HBX Mab in

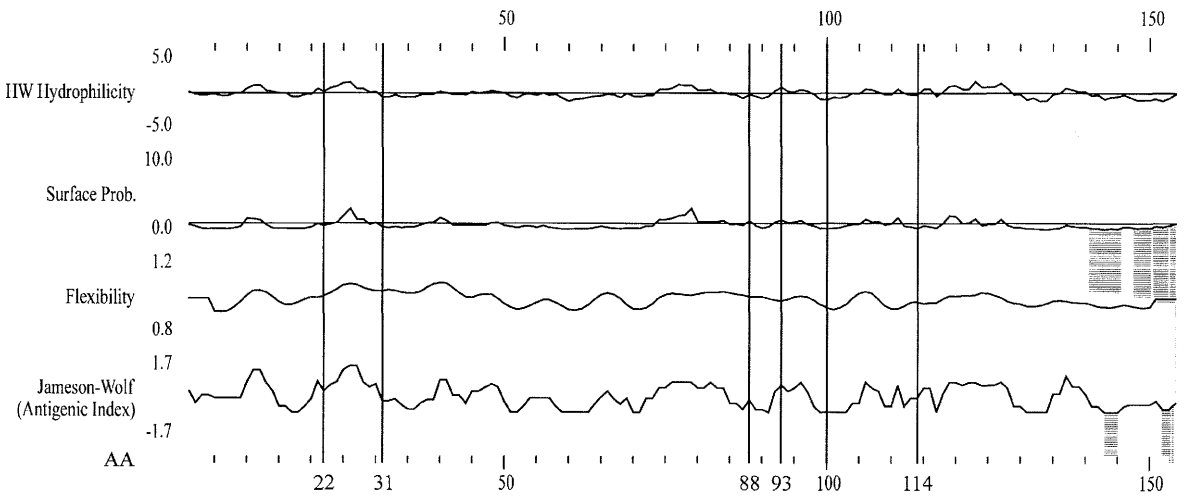

Fig. 1. Computer aided structure analysis of HBX. Peptides chosen on the basis of Jameson–Wolf antigenic index were tested negative for binding to our anti-HBX Mab (amino acids 22–31 and 100–114). The epitope localized by screening a phage displayed random peptide library is between amino acids 88 and 93.

Table 2
Deduced amino acid sequences of clones selected with anti-HBX Mab from a library of nine amino acid random peptides displayed on filamentous phage

PFLPKVLHK[a]
RMLPASLHL
RRLPEELHR
ARLPPSLHL
RNLPRALSP
RRLPSAFHP
LRSLHLVRG
SRPPSLFRP
RNVFLIRTE
GRVFFLVRP
DRNIYLVRQ

[a] Amino acids 86–94 of HBX.

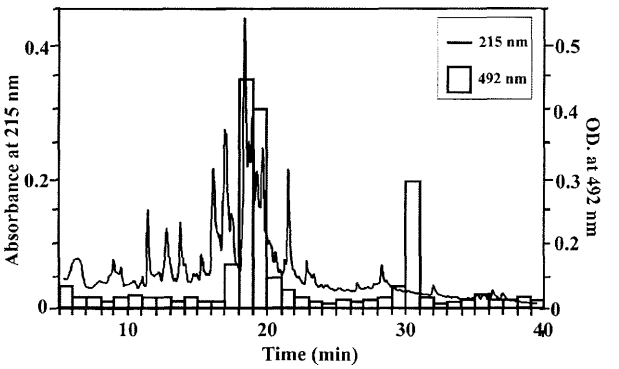

Fig. 2. RP-HPLC separation of peptide fragments after digestion with trypsin. UV-absorbance of the eluted fractions is indicated on the left vertical axis. Optical density following an ELISA with our anti-HBX MAb is indicated on the right vertical axis.

three fractions (Fig. 2). Fractions #18 and #19 showed high immunoreactivity and contained several peptides (with the molecular mass of 1471.7, 2524.0 and 2551.3 of the three main peaks, respectively). Only the first peptide could be assigned to an HBX tryptic fragment: amino acids 14–26 (DVLCLRPVGAESR). The nano-MS/MS spectrum of the double charged ion of this peptide confirmed the sequence. However, this result did not fit in the phage display prediction, and there was another fraction (#30) with positive result in ELISA. We could not identify any peptide in fraction #30 despite the positive ELISA result (Fig. 2).

In order to unequivocally confirm the result obtained by random peptide library we constructed three overlapping GST fusion peptides spanning amino acids 77–142 of the HBX (Table 1). The anti-HBX Mab recognized only the segment representing amino acids 77–116. No reaction was found when peptides containing sequence 96–135 and 116–142 were tested. According to the alignment of the segments only the sequence 77–95 can possibly contain the epitope bound by the anti-HBX Mab (Table 3). This calculation further supports the results of the random peptide library screen, which identifies the epitope sequence as LPxxLH (amino acids 88–93 of the HBX).

4. Discussion

The exact identification of epitope regions of antigens recognized by natural and genetically engineered (monoclonal) antibodies appears to be of crucial importance both in biotechnological research and routine diagnostics (Rapley, 1995; Davis et al., 1999; Milstein, 1999). Several techniques have been used for mapping the precise epitope structures. Various procedures based on overlapping synthetic peptide sequences are frequently used in the case of known primary antigen structures. These methods are sufficient in the majority of proteins and peptides; however, the correct mapping of non-linear epitopes is difficult (Helfrich et al., 1994). Mass spectrometric analysis represents a cost-effective, time saving, and well reproducible alternative (Zhang et al., 2002). The efficiency of these techniques can be enhanced by computer modeling and calculation of probable immunogenic regions on the antigen molecules.

Antigens with unusual physico-chemical features impair the efficiency of the application of classical immunoserological and spectrometric mapping techniques, and limit the value of computer aided structure analysis, as we found during the mapping of the epitope specificity of our anti-HBX monoclonal antibody.

Computer analysis of the molecular model suggested different possible immunogenic regions on HBX, however, the identified epitope is estimated to posses a relatively low predicted immunoreactivity (Fig. 1). This contradiction could be explained partially by the application of usual physico-chemical parameters on this highly hydrophobic protein.

Repeated reviewing of our data could indicate a possible explanation for the controversial results of our mass spectrometric analysis. The epitope sequence obtained by the random peptide library screen (LPKVLH 88–93) contains a

Table 3
Overlapping recombinant HBX fragments

Fragment	AA	ELISA
METTVKAQPFLPKVLHKRTLGLSVMSTTDLEAYFKDCLF	77–116	+
TLGLSVMSTTDLEAYFKDCLFKDWEELGEEIRLKVFVLGG	96–135	–
FKDWEELGEEIRLKVFVLGGCRHKLVC	116–142	–

AA: corresponding amino acids of the HBX protein; ELISA: reactivity with the anti-HBX Mab in ELISA.

trypsin cleavage site. If we consider fragments of the partially digested HBX, sequence 79–95 has one such site, sequence 79–96 has two and sequence 79–112 has three missed cleavage sites, respectively. The hydrophobicity of these fragments calculated by the method of Guo et al. (1987), Browne et al. (1982), Rekker (1977) is higher than that of peptide 14–26. Higher hydrophobicity results in a longer retention time in reverse-phase chromatography. Although the digestion of proteins by trypsin is usually complete in the applied process, partial cleavage may always occur. There was no UV absorbance around 30 min, and even MS measurement could not detect any peptide in this fraction (Fig. 2). The fraction #30 may contain any of these peptides with missed digestion site. As our Mab proved to be strongly reactive against the in vivo expressed HBX in pathologic hepatocytes (chronic active hepatitis, chronic persistent hepatitis, cirrhosis, primary hepatocellular carcinoma) in paraffin embedded tissue sections, it could be sufficiently sensitive to detect the minute amount of partially digested peptide in fraction #30.

Application of molecular biological techniques offered new possibilities in antigen mapping. The combination of phage displayed peptide libraries with immunoserological determination of the expressed antigen fragments and antibodies results a new potential. The fine epitope specificity of anti-HBX Mab verified by phage display technique was confirmed by immunoserological control on synthetic peptide antigen and on overlapping recombinant antigen constructs.

In summary, all mapping techniques require regular control measurements using independent assays in a complex application of current molecular biological and immunological techniques. Our method seems to be useful for the analysis of epitope specificity of natural serum antibodies reacting with HBX molecule in the near future. These investigations would help to clarify the prognostic relevance of different antigenic sites on HBX protein.

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Validation of in silico prediction by in vitro immunoserological results of fine epitope mapping on citrate synthase specific autoantibodies

Z. Nyárády[a,b], T. Czömpöly[a], Sz. Bősze[c], G. Nagy[a], Á. Petrohai[d], J. Pál[a,e], F. Hudecz[c,f], T. Berki[a], P. Németh[a,*]

[a] *Department of Immunology and Biotechnology, University of Pécs, Szigeti út 12, H-7643 Pécs, Faculty of Medicine, Hungary*
[b] *Department of Oral and Maxillofacial Surgery, University of Pécs, Dischka Gy. u. 5, H-7621 Pécs, Hungary*
[c] *Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Pázmány P. s. 2, H-1518 Budapest, Hungary*
[d] *Department of Cardiovascular Surgery, Semmelweis University, Városmajor u. 68, H-1122 Budapest, Hungary*
[e] *Department of Neurosurgery, University of Pécs, Rét u. 3, H-7623 Pécs, Hungary*
[f] *Department of Organic Chemistry, Eötvös L. University, Pázmány P. s. 1A, H-1518 Budapest, Hungary*

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Abstract

In silico antibody–antigen binding predictions are generally employed in research to rationalize epitope development. These techniques are widely spread despite their technical limitations. To validate the results of these bioinformatic calculations evidence based comparative in vitro studies are necessary.

We have used a well-conserved mitochondrial inner membrane antigen–citrate synthase to develop a model for comparative analysis of the predicted and the immunoserologically verified epitopes of circulating autoantibodies. Epitopes were predicted using accepted tools: the *GCG Wisconsin* package and *TEPITOPE 2000*. An overlapping multipin ELISA assay – covering 49% of the citrate synthase molecule – was developed to map autoantibody epitopes of individuals (healthy, systemic autoimmune, and heart transplanted) in different immunopathological conditions.

From the 40 synthesized decapeptides 34 were predicted in silico and 27 were validated in vitro. Thirty-two percent of epitopes were recognized by majority of sera 47% by at least one sera. False positive predictions were 21%. There was major difference in the recognized epitope pattern under different immunopathological conditions.

Our results suggest that special databases are needed for training and weighing prediction methods by clinically well-characterized samples, due to the differences in the immune response under different health status. The development of these special algorithms needs a new approach. A high number of samples under these special immunological conditions are to be mapped and then used for the "fine tuning" of different prediction algorithms.

Keywords: Autoimmunity; In silico epitope prediction; Autoantibody; Citrate synthase; Multipin ELISA; Immunoserology; Heart transplantation

1. Introduction

The diagnosis of autoimmune diseases shows an increasing tendency in highly developed countries and causes growing socio-economic problems. We have no specific therapy or prevention strategy against them, and there is no common theory for their pathomechanism. Detailed analysis of the targeted epitopes by autoreactive T-cells and autoantibodies has an outstanding importance in immunopathological research and development as well as in the design of novel diagnostic tools, therapeutic drugs or vaccines (Laver et al., 1990;

* Corresponding author. Tel.: +36 72 536290; fax: +36 72 536289.
E-mail address: peter.nemeth@aok.pte.hu (P. Németh).

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Sturiniolo et al., 1999; van Regenmortel, 2000; Németh et al., 2004).

Efficient fine molecular mapping techniques are required for the better understanding of the relation between the biomolecules' structure and functions (Pellequer et al., 1991). State-of-the art microanalytical techniques are able to analyze distinct submolecular characters and combine them with biological functions; however, the extended applications of conventional laboratory methods are time and resource consuming. The development of computation power and algorithms to analyze biological data increased the efficiency of laboratory research and diagnostics during the last decade, and by the use of in silico research tools, the development of drugs or vaccines became more efficient and cost effective (van Regenmortel and de Marcillac, 1988; Peri et al., 2001).

Various algorithms are available for calculation of immunogenity index and predicting epitopes on antigens for further immunological studies (Hopp, 1986; van Regenmortel, 2000). Most of these techniques involve calculations based on scales of values for the twenty amino acids reflecting their likelihood of occurrence at the surface of proteins or as part of secondary structures, such as helices or beta-bends. The most successful of these algorithms use scales related to the water solubility of the individual amino acids. In particular, the highest success rates are obtained by using hydrophilicity scales that emphasize the charged and polar amino acids, but are not overly selective for either positive or negative charges. Such a method can correctly pinpoint major antigenic sites on the proteins of most well-characterized infectious organisms (Hopp, 1986).

Drug design – including vaccine development – needs efficient assistance in selecting the possible candidate molecules for novel and improved drugs in all fields of treatment and in the prevention of infectious diseases caused by resistant microorganisms, the hazard of bio-terrorism, or bio-therapies for cancer, organ transplantation as well as autoimmune diseases (Cohen and Young, 1991; Schawarz and Cohen, 2000; Németh et al., 2004). By predicting epitope one would be able to develop specific monoclonal antibodies with short peptide immunization raising antibodies cheaper and quicker (van Regenmortel, 1986; van Regenmortel, 1989; Schwede et al., 2000).

The genome-based vaccine research opened new potential issues for the selection of the targeted antigens using in silico predictions for analysis of the genomes and using set of recombinant techniques to express the potential antigen fragments. (Serruto et al., 2004; Masignani et al., 2004) Laboratory diagnostics, drug design and vaccine developments are intensively growing areas of current medical biotechnology where computing techniques play an essential role (Guex et al., 1999; Andersen et al., 2000; Masignani et al., 2004). However, the evidence based verification of these bioinformatically predicted epitopes targeted by autoantibodies has been the focus of recent studies only (Peri et al., 2001).

Our major goal was to compare and evaluate the data of in silico predictions with the results of in vitro immunoserological measurements in a well-characterized model: fine epitope specificity of circulating autoantibodies was determined and analyzed on the mitochondrial citrate synthase (CS) enzyme to assess the value of predicted and immunoserologically verified epitopes under normal and different pathological conditions.

2. Materials and methods

2.1. B-cell epitope prediction

The human mitochondrial CS sequence (EC 2.3.3.1; GenBank accession no. PIDO75390; Goldenthal et al., 1998) used for prediction analysis was obtained from the protein sequence database (Benson et al., 1999; http://www.ncbi.nlm.nih.gov/Entrez/index.html).

To localize the potentially antigenic sites, we have predicted β-turns of CS protein segments using the Chou-Fasman secondary structure prediction method (Chou and Fasman, 1978; Prevelige and Fasman, 1990) and hydrophobicity predictions by Eisenberg et al. (1990). In both cases, windows of seven amino acids were used in MS Excel applying the original matrices of the authors. Segments with high probability of β-turn secondary structure ($P_{\beta\text{-turn}} > 1$) and low probability of hydrophobicity ($P_{\text{hydrophobicity}} < 0$) were selected. We also used the *PepPlot, PeptideStructure* and *PlotStructure* programs of the *GCG Wisconsin Package* (http://www.gcg.com) for surface probability ($P_{\text{surface}} > 1$), Jameson-Wolf antigenity index ($P_{\text{JW antigenity}} > 1$) (Jameson and Wolf, 1988) and hydrophilic regions ($P_{\text{HW hidrophilicity}} > 1$) (Hopp and Woods, 1981) to fine tune our prediction results.

Swiss-Model Automated Protein Modelling Server (http://www.embl-heidelberg.de/predictprotein) was used to obtain a 3D model of human CS, based on homology *E. coli* CS (EC 4.1.3.7, Brookhaven Protein Data Bank No. 1K3P; Nguyen et al., 2001) (Peitsch, 1996; Guex et al., 1999; Berman et al., 2000; Schwede et al., 2000; Peitsch, 2002).

2.2. T-cell epitope prediction

T-cell epitope prediction was done by a virtual matrixes method formed by assigning and combining pocket-specific quantitative binding values derived from one HLA allele to other alleles via HLA sequence comparison, using the *TEPITOPE 2000* (Juergern Hammer, Vaccinome 1999) program (Hammer et al., 1994; Sturiniolo et al., 1999). Scanning was performed with all the 25 HLA-II alleles available in the program (DRB1*0101; DRB1*0102; DRB1*0301; DRB*0401; DRB1*0402; DRB1*0404; DRB1*0410; DRB1*0421; DRB1*0701; DRB1*0801; DRB1*0802; DRB1*0804; DRB1*0806; DRB1*1101; DRB1*1104; DRB1*1106; DRB1*1107; DRB1*1305; DRB1*1307; DRB1*1311;

DRB1*1321; DRB1*1501; DRB1*1502; DRB1*0101). HLA binding pockets were weighed evenly. A 1% threshold value was used to select the high specificity probable epitopes.

2.3. Serum samples

Serum samples, from healthy individuals collected during a 5-year period (63 healthy Hungarian, 51 healthy British and 176 healthy Finnish adult blood-donors, and 44 serum samples from healthy infants), samples of patients with systemic autoimmune diseases (326 clinically well-documented cases) and sera of 52 heart transplant patients were analyzed. Serum samples were stored at −70 °C before examination. The age and sex ratios of patient groups were matched.

2.4. Immunoserological detection of anti-CS autoantibodies by ELISA

An indirect ELISA was used to detect anti-CS autoantibodies from human sera: CS from porcine heart (Sigma) as antigen was incubated for 12 h at +4 °C followed by 60 min at 37 °C on 96-well polystyrene plates (NUNC, France). Following the saturation of non-specific binding sites with 0.5% gelatine (Sigma, USA) in PBS (pH 7.3), three washing steps with PBS containing 0.1% Tween 20 (Sigma, USA) were performed. The triplicated samples were incubated at 1:100 dilutions (diluted in washing buffer) for 60 min. Finally, the plate was incubated with anti-human IgG-HRPO or anti-human IgM HRPO secondary antibodies (Dako, Denmark) for 60 min. The color reaction was developed with *o*-phenylenediamine (Sigma, USA), and measured on Dynatech MR7000 (USA) microphotometer at $\lambda = 492$ nm (OD, optical density). The results were compared to controls containing no CS but all the other components, no primary sera, and no secondary anti-human IgG-HRPO or anti-human IgM HRPO.

2.5. Multipin peptide synthesis

Forty decapeptides overlapping five amino acid residues were synthesized, corresponding to the nine regions predicted, on β-alanyl-glycine functionalized polyethylene pins (Mimotopes, Australia) with Fmoc/tBu chemistry according to Geysen's method (Geysen et al., 1984). We used ^{t}Bu (Thr, Ser, Tyr), O^{t}Bu (Asp, Glu), Trt (Cys, His, Gln, Asn), Pbf (Arg) and Boc (Lys, Trp) as side chain protecting groups. Briefly, the Fmoc α-amino protecting group was removed with 20% piperidine/DMF (v/v). Coupling was performed with DIC/HOBt methodology and monitored with bromophenol blue added to the coupling mixture (Krchnák et al., 1988). Peptides were acetylated at the *N*-terminus, then the side chain protecting groups were removed with TFA – EDT – anisole 38:1:1 (v/v/v), but the unprotected peptides remained covalently attached to the pins. Two control peptides were also prepared: PLAQGGGGGG and GLAQGGGGGG.

2.6. Immunoserological epitope mapping of anti-CS autoantibodies using overlapping decapeptides in multipin system

Ninety-six-well polystyrene ELISA plates (NUNC, France) and pin-bound peptides were the hardware of epitope mapping. All steps were preformed with agitation. First, we kept the pins in PBS containing 0.5% gelatine for 60 min at room temperature for saturation of non-specific binding sites. Then the pins were washed three times with PBS containing 0.1% Tween 20. The serum samples and controls were added to the plates in the dilution of 1:100 (diluted in PBS containing 0.5% gelatin and 0.1% Tween-20). After 60 min of incubation, the pins were washed again for 3 × 10 min with PBS containing 0.1% Tween-20. Finally, pins were incubated with anti-human IgG-HRPO or anti-human IgM HRPO secondary antibodies for 60 min at room temperature. After washing (3 × 10 min) the color reaction was developed with *o*-phenylenediamine and measured on iEMS MF micro photometer (ThermoLabsystem, Finnland) at 492 nm (OD). The data were analyzed with Ascent Software V.2.4.2 (ThermoLabsystem, Finnland).

2.7. Homology searches

In addition to the antibody binding experiments, we have compared the sequence of epitopes of human CS with that of proteins present in the non-redundant protein database by BLASTP2 program. (Expect = 20,000; Matrix PAM30; Gap cost Existence: 9 Extension: 1; Word size = 2, database size as of September 2004 = 688,443,072.) Immunologically relevant hits showed homology with proven or hypothetical CS, but only negligible similarity was found between CS and non-CS proteins (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

3. Results

The complete epitope mapping of the well-conserved mitochondrial inner membrane CS enzyme using autoantibodies obtained under normal and pathological conditions was made, and the results of in silico antigenity prediction with the in vitro immunoserological results were compared.

3.1. Results of B-cell epitope prediction

Assuming that antibody epitopes are usually found at or near to hydrophilic β-turn parts of proteins, fifteen segments with high probability of β-turn secondary structure ($P_{\beta\text{-turn}} > 1$) and low probability of hydrophobicity ($P_{\text{hydrophobicity}} < 0$) were considered for selection. These segments cover the following sequences: 51–78, 85–91, 103–119, 127–132, 138–161, 176–195, 217–239, 244–250, 261–269, 281–285, 302–309, 320–331, 354–364, 390–398, 409–414, 448–463 (Fig. 1).

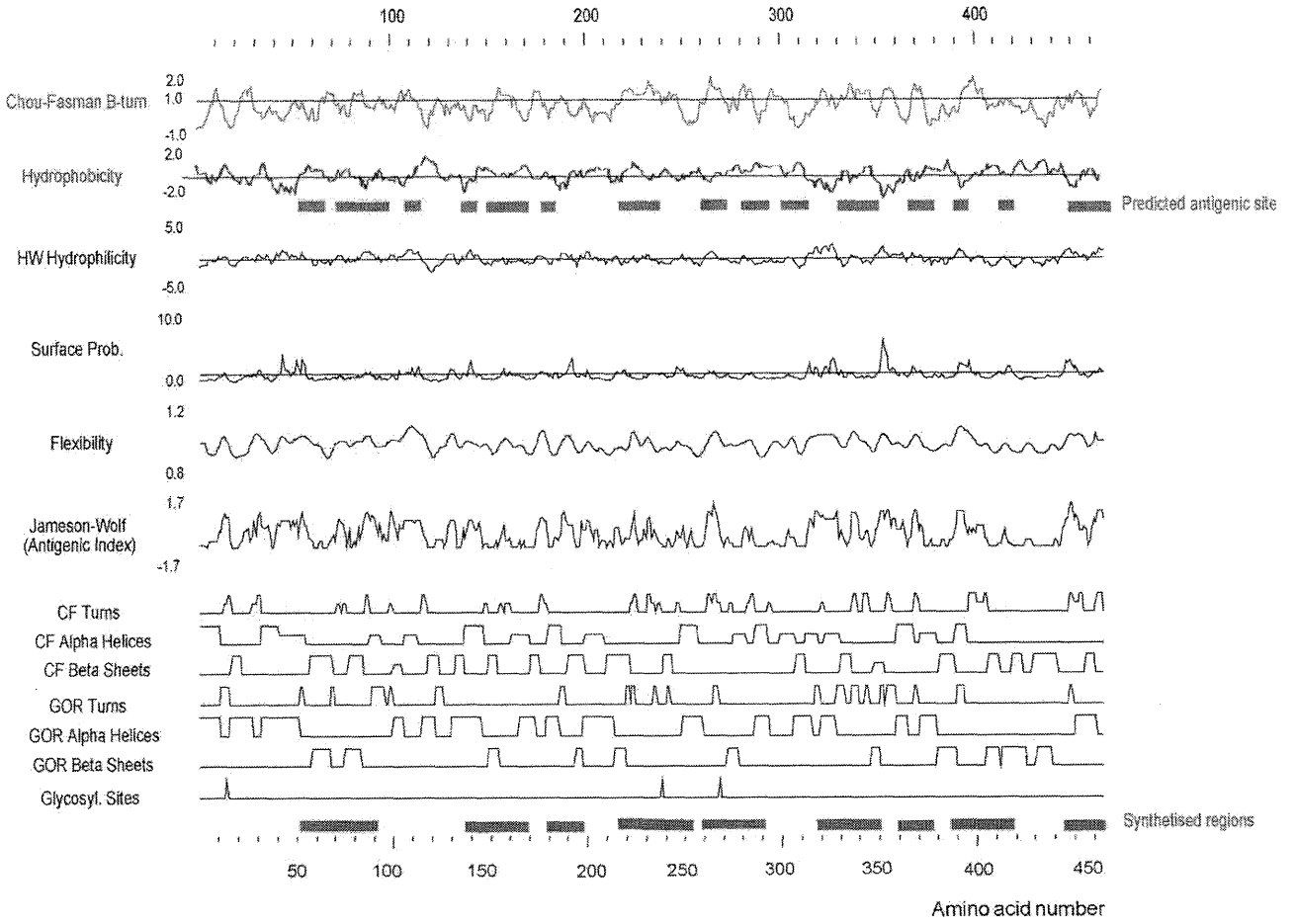

Fig. 1. The analysis of CS by the most commonly used prediction methods for immunogenity are plotted in the figure. Chou-Fasman β-turn prediction (purple; $P_{\beta\text{-turn}} > 1$), Hydrophobicity (green; $P_{\text{hydrophobicity}} < 0$); predicted antigenic sites (blue) and synthesized overlapping sequence used in vitro (red).

Considering the results of 3D homology modeling, two of the fifteen segments were rejected. Regions 103–119 and 302–309 had only very limited or no accessibility for antibody binding, as they are located not on the surface of the molecule (Fig. 2). Based on scores derived from the prediction and modeling results, the following nine segments were selected for further studies: 51–90 (I), 124–163 (II), 176–195 (III), 216–250 (IV), 261–285 (V), 320–339 (VI), 354–373 (VII), 390–414 (VIII), and 447–466 (IX). For immunoserological validation of predicted epitopes, a multipin system with set of overlapping decapeptides covering regions I–IX were prepared by solid phase chemistry (Table 1).

3.2. Results of T-cell epitope prediction

Amino acid sequences 18–29, 35–88, 92–115, 119–131, 139–165, 195–237, 254–266, 306–319, 361–393, and 406–446 were predicted by binding to at least one of virtual HLA allele. Nine segments, showing binding potential to more than 10 virtual HLA alleles were regarded as highly potential for T-cell epitopes (18–29, 57–83, 91–103, 119–131, 201–215, 254–266, 306–319, 373–393 and 415–427). 3D modeling showed that two of these regions are located on α-helices positioned in the inner part of the protein (Fig. 2). It is interesting to note, that four of the nine segments partly overlap with the predicted B-cell epitope regions: 57–60, 57–79, 255–266, 306–319, and 415–427. One of these overlapping sequences (306–319) is located also in the intramolecular space. Three T-cell predicted highly potential sequences on the surface of the molecule were just adjacent to predicted B-cell epitopes (T|B: 91–103|103–119, 119–131|103–119, 415–427|409–414).

3.3. Results of ELISA

Sera having an OD value at least two standard deviation (2 S.D.) higher than the average of measured OD of sera in the group were selected as positive. The positive anti-CS IgG cases were 2–4% of all subgroups, except among heart transplant patients, where 7% of all cases were positive. IgM sera were positive in 7–12% of all cases, except the autoimmune patients, where 24% of all cases were positive

Table 1
In silico selected regions and synthetic overlapping decapeptides used for studies in the multipin system

```
I.
(51) FRQQHGKTVVGQITVDMMYGGMRGMKGLVYETSVLDPDEG (90)
     FRQQHGKTVV          KMRGMKGLVY
          GKTVVGQITV          EGLVYETSVL
               GQITVDMMYG          DTSVLDPDEG
                    GMMYGGMRGM

II.
(124) VTGCIPTEEQVSWLSKEWAKRAALPSHVVTMLDNFPTNLH (163)
     VTGCIPTEEQ          RAALPSHVVT
          PTEEQVSWLS          SHVVTMLDNF
               VSWLSKEWAK          MLDNFPTNLH
                    KEWAKRAALP

III.
(176) NSESNFAQAYARGISRTKYW (195)
     NSESNFAQAY
          FAQAYARGIS
               ARGISRTKYW

IV.
(216) IYRNLYWEGSGIGAIDSNLDWSHNFTNMLGYTDHQ (250)
     IYRNLYWEGS          WSHNFTNMLG
          YWEGSGIGAI          TNMLGYTDHQ
               GIGAIDSNLD
                    DSNLDWSHNF

V.
(261) IHSDHEGGNVSAHTSHLVGSALSDP (285)
     IHSDHEGGNV
          EGGNVSAHTS
               SAHTSHLVGS
                    HLVGSALSDP

VI.
(320) GKDVSDEKLRDYIWNTLNSG (339)
     GKDVSDEKLR
          DEKLRDYIWN
               DYIWNTLNSG

VII.
(354) DPRYTCQREFALKHLPNDPM (373)
     DPRYTCQREF
          CQREFALKHL
               ALKHLPNDPM

VIII.
(390) EQGKAKNPWPNVDAHSGVLLQYYGM (414)
     EQGKAKNPWP
          KNPWPNVDAH
               NVDAHSGVLL
                    SGVLLQYYTV

IX.
(447) ERPKSMSTEGLMKFVDSKSG (466)
     ERPKSMSTEG
          MSTEGLMKFV
               LMKFVDSKSG
```

Light-shaded background in the synthesized region shows predicted epitopes. Dark-shaded background in decapeptide sequence shows amino acids differing from the selected regions, due to the limitations of the multpin system. *Italics* represents the possible HLA-binding sites.

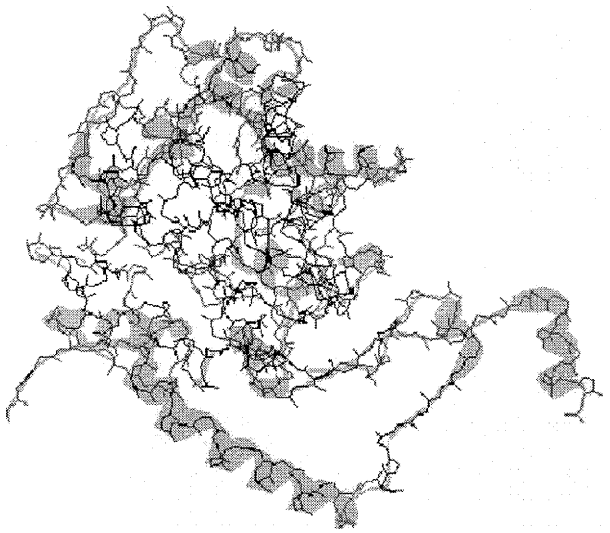

Fig. 2. Location of predicted linear B-cell epitopes on 3D homology model of mammalian CS. Based on Prevelige-Fasman prediction and hydrophobicity calculations, sixteen regions of different length, 4–23 amino acid residues were selected (backbone in red). Potentially antigenic sites according to the tertiary structure model are represented (green ribbons).

(Table 2). Serum samples found positive during the simple binding ELISA test were used for the further epitope mapping.

3.4. Immunoserological epitope mapping in multipin system

Results of epitope mapping are summarized in Table 3. Major differences were found in the recognition pattern of different types of sera. Differences in epitope pattern of healthy and heart-transplanted patients have been published recently (Petrohai et al., 2005). The results of detailed immunopathological analysis on the differences between the results of the autoimmune and healthy samples are under publication (Czömpöly et al., under publication).

The examined 40 decapeptides (synthetic epitope) covered 49% of the whole CS molecule. From 40 decapeptides 23 (58%) were recognized by more than one group of sera; 8 (19%) were recognized only by one group of sera, while 9 (23%) did not had any recognition.

From the 40 synthetic decapeptides 34 were predicted in silico for good immunological recognition, and 27 (79%) of

Table 2
Distribution of anti-citrate synthase positive sera

Description of group (no. of serum samples)	IgG (%)	IgM (%)
Hungarian blood-donors (63)	3	10
British blood-donors (51)	4	12
Finnish blood-donors (176)	3	9
Healthy infants (44)	2	7
Systemic autoimmune patients (326)	3	*24*
Heart transplanted patients (52)	*7*	12

Table 3
Epitopes recognized by IgG and IgM autoantibodies of different groups of patient sera

Predicted region	AA position	Decapeptide	IgG healthy	IgM healthy	IgG autoimmune	IgM autoimmune	IgG transplanted	IgM transplanted
I (51-91)	51-60	*FRQQHGKTVV*	+	+	+	+	+	
	56-65	*GKTVVGQITV*	+	+				
	61-70	*DQITVDMMYG*				+		
	66-75	*GMMYGGMRGM*	+	+	+			++
	71-80	*KMRGMKGLVY*	+		+			
	76-85	*EGLVYETSVL*						
	86-91	*DTSVLDPDEG*						
II (124-163)	124-133	*VTGCIPTEEQ*				++		
	129-138	*PTEEQVSWLS*				+++		
	134-143	VSWLSKEWAK	+	++	++	+++	++	++
	139-148	KEWAKRAALP	+	+	++	+	+++	
	144-153	RAALPSHVVT	+	+	+	+	+	++
	149-158	SHVVTMLDNF		+	+		+	+
	154-163	MLDNFPTNLH					++	+
III (176-195)	176-185	NSESNFAQAY						
	181-190	FAQAYARGIS		+	+	++		++
	186-195	ARGISRTKYW	+	+++	+++	+	++	
IV (216-250)	216-225	*IYRNLYWEGS*	+	+++	+	+++	+++	++
	221-230	*YWEGSGIGAI*	+	+	+	+++	+++	+
	226-235	*GIGAIDSNLD*				+	++	
	231-240	*DSNLDWSHNF*				+		
	236-245	WSHNFTNMLG	+	+		+++	+	+++
	241-250	TNMLGYTDHQ		+				+
V (261-285)	261-270	*IHSDHEGGNV*						
	266-275	EGGNVSAHTS						
	271-280	SAHTSHLVGS		+			+	++
	276-285	HLVGSALSDP		+				
VI (320-339)	320-329	GKDVSDEKLR						
	325-334	DEKLRDYIWN				+		
	330-339	DYIWNTLNSG		+	+	+++	++	
VII (354-373)	354-363	DPRYTCQREF		+	+	+++		+
	359-368	CQREFALKHL	+		+	++	+	
	364-373	*ALKHLPNDPM*				+		
VIII (390-414)	390-399	EQGKAKNPWP						
	395-404	KNPWPNVDAH						+
	400-409	NVDAHSGVLL						
	405-414	SGVLLQYYTV				+		
IX (447-466)	447-456	ERPKSMSTEG			+			
	452-461	MSTEGLMKFV						
	457-466	LMKFVDSKSG	+		+			

Shaded background represents predicted epitopes. (AA: amino acid; +: 0%–25%, ++: 25%–50% and +++: 50%–100% of sera bind to the given decapeptide.)

them were confirmed by immunoserological measurements. Eleven decapeptides (32%) were recognized by the majority of sera, 16 (47%) by at least one. Seven (21%) decapeptides were predicted in silico but not validated in vitro. There were six peptides, which were not predicted but synthesized, three were not and three were recognized by autoantibodies. One unpredicted synthetic peptide (330–339 amino acid sequence) had strong reactivity with the different groups of sera (Table 3.).

The results of the homology searches of the in silico predicted and in vitro analyzed epitopes are presented in Table 4. The comparison of binding data obtained from ELISA experiments and scores from this analysis clearly show that all peptides recognized by autoantibodies possess high score hits to the conserved parts of CS first, and some well-conserved – mostly bacterial – hits were found as well.

4. Discussion

To make a difference between "physiological" and "pathological" self-reacting antibodies is not only a theoretically relevant question, but it has major clinical consequences in diagnosis, treatment, monitoring, and prevention of autoimmune diseases. Factors regulating and determining the tolerance of the most important self-structures are studied intensely. The postulation of "immunological homunculus theory" opened new aspects in autoimmunity research during the last decade (Cohen and Young, 1991; Cohen, 1992; Schawarz and Cohen, 2000). Several molecular biological and immune physiological studies were initiated based on this theory, however, the role of physiological autoimmunity in the active tolerance and in the occurrence of different autoimmune diseases remain mostly unclear. Only complex studies

Table 4
Homology search results. All subsequences recognized by autoantibodies possess high score hits to the conserved parts of CS first, and some well-conserved – mostly bacterial – hits were found as well

CS subsequences	Description
51–78	Conserved CS
51–60	Conserved CS, DNA-damage protein
61–71	Conserved CS, Ser-protease inhibitor, Enzymes
71–79	Conserved CS
85–91	Conserved CS
127–132	Conserved CS, bunch of bacterial proteins
138–161	Conserved CS, DNA-damage dependent protein, DNA-repair protein
176–195	Conserved CS
187–197	Conserved CS, tRNA-synthase (bact, Ricketsia, Neiseria, etc.), P1 protein (bacterial, Pseudomonas)
217–239	Conserved CS, bacterial proteins
221–231	Conserved CS, several bacterial proteins
236–245	Conserved CS, NADH-dehidrogenase
241–250	Conserved CS
261–270	Conserved CS
280–285	Conserved CS, conserved enzymes
320–329	Conserved enzymes
330–339	Conserved CS, conserved enzymes
354–363	Conserved CS, conserved enzymes
359–368	Conserved CS
364–373	Conserved enzymes
390–404	Conserved CS
405–414	Conserved CS
448–463	Conserved CS

using molecular and cellular biological experiments and clinical epidemiological surveys are able to clarify the alteration in the most important regulatory pathways of autoimmunity.

Analyzing the fine molecular structures of the targeted epitopes is important both for the prognostic values and for the further therapeutic consequences in different autoimmune diseases. In our experiments, we investigated the immunoreactivity of a genetically conserved antigen: CS is one of the earliest proteins with basic biological functions in the citric acid cycle and subsequent ATP generation. Autoantibody production against this antigen was analyzed in healthy individuals, autoimmune, and heart transplant patients during our previous studies. Remarkable evidences were found for differences in epitope pattern and the participation of anti-CS autoantibodies in the formation of physiological and pathological autoimmune reactions (Németh et al., 2004; Petrohai et al., 2005; Czömpöly et al., under publication). However, the results of the in silico epitope predictions performed earlier were not fitting to the measured data in some cases. This finding initiated a systematic comparative analysis between in silico predictions and in vitro immunoserological measurements. The CS study was an appropriate model for the validation of bioinformatic predictions.

The verification of bioinformatically predicted epitopes could be achieved by the use of synthetic peptides and appropriate antibodies. An overlapping peptide library was created and applied according to our prediction data.

Theoretically, the entire accessible surface of a molecule may be regarded as a large number of overlapping epitopes of a size of roughly 700 $Å^2$ (Benjamin et al., 1984). Not all of these potential epitopes are relevant immunologically. The immunological recognition and the antigen specific responses depend on the recipient immune system and on the antigen's actual conformation (Attasi, 1984). Most of the epitopes are assembled or conformational, only some of them are continuous ones (van Regenmortel and de Marcillac, 1988; van Regenmortel, 1989). The most precise way of identifying an epitope is either X-ray crystallography or NMR of the given peptide and antibody bound to it. Both approaches have their technical limitations as they require specific criteria for deciding which residues of the antigen are in contact with the paratope and are functionally part of the epitope (van Regenmortel, 1989).

The limitations in computational power, our knowledge of epitope structure of proteins and the paratope–epitope binding as well as the lack of reliable tertiary structure data of proteins focused the attention on epitope prediction from primary structure of proteins (van Regenmortel, 2000). These algorithms have the limitation of predicting potential continuous linear epitopes only, which certainly will not represent all the recognized immunologically active epitopes of an individual in its given state.

Short synthetic peptide sequences are suitable tools for exploring the molecular subregions (epitopes) of larger proteins recognized by antibodies (Lerner, 1982; Lerner, 1984).

The multipin overlapping peptide synthesis combined with ELISA is an ideal way of searching for continuous linear epitopes. This 96-position array format provides a simple and cost effective entry point into multiple parallel organic syntheses as our CS experiment. The chosen 10 amino acid long peptides are suitable for simultaneous synthesis of a large number of compounds without the requirement for complex filtration systems. These decapeptides are able to mimic the linear parts of the original protein (Maeji et al., 1995).

For the majority of proteins known today, the only available structural information is their primary amino acid sequence, usually deduced from their nucleotide sequence of the corresponding gene. Many attempts were made to utilize this information to predict the position of continuous epitopes. Parameters such as hydrophilicity, mobility and accessibility of short segments of polypeptide chains were been correlated with the location of continuous epitopes in a few well-characterized proteins. This led to the development of several prediction scales to locate continuous epitopes (van Regenmortel, 1986; Hopp, 1986). For our study, we had chosen some well-proven, widely accepted and available methods, collected in the *GCG Wisconsin Package*.

The Chou-Fasman secondary structure prediction method is an accepted approach, which proved to be 75% efficient in prediction of continuous epitopes (Hopp, 1986; Prevelige and Fasman, 1990). The Hopp and Woods hydrophilicity approach is one of the most efficient methods by predicting up to 100% of epitopes among certain conditions (Hopp

and Woods, 1981; Hopp, 1986; Pellequer et al., 1991). The antigenity index, proposed by Jameson-Wolf, combines profiles of hydrophilicity, accessibility, flexibility and secondary structure. This method is able to predict continuous epitopes with 90% probability (Jameson and Wolf, 1988; Thornton et al., 1986).

Advances in understanding antigen presentation at the molecular level accelerated the development of computer models capable of predicting T-cell epitopes (Hammer et al., 1994; Honeyman et al., 1998; Andersen et al., 2000). Interaction of peptide fragments with HLA molecules was characterized thoroughly. HLA peptide binding is proved to be the major bottleneck in the selection of epitopes, as indicated by the finding that most peptide sequences lack the capacity to interact with HLA molecules. The T-cell epitope prediction approach with virtual HLA II matrices incorporated into *TEPITOPE*, like quantitative matrices, provides a detailed model in which the contributions to binding of each amino acid (ligand) with each pocket/position (HLA binding cleft). *TEPITOPE* proved to be capable of recognizing a wide variety of HLA II epitopes (Hammer et al., 1994; Sturiniolo et al., 1999; de Groth and Rothman, 1999).

The in silico analysis coincided frequently, but not always, with the immunoserologically determined epitopes. Synthesized regions covered 49% of CS, nearly the whole immunologically accessible region of the molecule. Individual sera groups recognized 32–50% of all predicted epitopes with remarkable differences between the healthy, autoimmune or heart transplanted groups. Antibodies of various groups of sera recognized 79% of predicted epitopes. This is comparable to the literature, where 60–100% of all epitopes are identified by in silico methods (Hopp, 1986; Pellequer et al., 1991). Predictions under different immunological conditions (individual sera groups) differ from the ideal data in the literature. This is because, methods currently employed in attempts to identify antigenic determinants and other features of proteins are currently trained under "healthy" immunological conditions, and tuned for recognizing the immunologically possible epitopes. From the 34 predicted epitopes, 27 were validated immunoserologically. There were seven false positive predictions (these were predicted but not validated), i.e. 21% of all predicted epitopes. In the detailed analysis of individual sera groups this incidence increases drastically to 50–68%. The variability between the analyzed groups can be explained by the differences in the immune response under different health status. The immunological conditions of the host immune system can determine the recognition pattern of the selected epitopes.

Our results suggest that special databases are needed for training and weighing prediction methods by clinically well-characterized samples. The development of these special algorithms needs a new approach. A high number of samples under these special immunological conditions are to be mapped and then used for the "fine tuning" of different prediction algorithms. This can introduce a new era of post-prediction in bioinformatics.

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