PhD thesis

Epitope mapping of natural antibodies

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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 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 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, secreted proteins including mannose binding lectin, and the third functional group of Toll-like receptors (TLRs).

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.

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.

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. 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.

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.

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. 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. 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.

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. They are long lived in vitro, can be forced with phorbol esters to proliferate, and they could not be activated through BCR crosslinking. The immunoglobulin repertoire of B1 B cells is restricted in the number of immunoglobulin genes used, it is dominated by rearrangement of J-proximal V genes and has significantly fever N insertions than the repertoire of B2 cells.

Functions of B1a cells include the participation in the early phases of immune responses 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. 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. These antibodies can recognize genetically conserved sequences of pathogens and may serve in the first line of immune defense during an infection. 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. Most nAAbs belong to the IgM or IgG isotype and show polyreactivity with a broad range of affinities for the recognized epitopes. 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, aid the clearance of apoptotic cells, possess anti-inflammatory effects and contribute to the maintenance of immune homeostasis. 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 it is probable that specificities with self non-self cross reactivity exist.

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.

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; 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 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 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 were coated with CS, malate dehydrogenase (MDH; EC 1.1.1.37) and pyruvate dehydrogenase (PDH; EC 1.2.4.1) from porcine heart in 0.1M bicarbonate buffer. Following the saturation of non-specific binding sites with 0.5% gelatin , serum samples were incubated in triplicates at 1:100 dilutions in washing buffer for 60 min. Finally, the plate was incubated with HRPO conjugated anti-human-IgA, or -IgG or –IgM specific secondary antibody for 60 min. The reaction was developed with *o*-phenylenediamine , and measured 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.

Affinity purification of sera on CS

CS from porcine heart was coupled to cyanogen-bromide activated sepharose 4B 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

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 as antigens.

Reactivity with E.coli CS was tested with pin-bound overlapping decapeptides as described previously.

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 and Sm, RNP, SSA, SSB, Scl-70, Jo-1, CCP, thyreoglobulin, glomerular basal membrane specific autoantibodies.

Immunocytochemistry

Immunocytochemistry with affinity purified anti-CS sera from SLE patients was performed on Hep-2 cells using the Anafluor kit. 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) was added and incubated for 30 min. After washing the bound antibodies were visualized with a goat anti-rabbit FITC conjugate.

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. 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. 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 and blocking, 10^{10} phage was added and incubated for 2h. The plate was washed and the bound phage were

eluted and following neutralization 10 ml of E.coli XL1-Blue was infected and plated on LB agar plates containing ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with 10^{11} M13KO7 helper phage. After an overnight incubation at 37°C, phage were precipitated. 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

Three overlapping parts of the HBX gene 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 glutathione-S-transferase (GST)-glutathione affinity system with modifications.

Flow cytometry

The inhibitory effect of phages displaying the putative IBL-8 epitope was determined using phages purified with 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 ($5x10^6$ cells/ml) was added. After 20 mins on ice, the cells were washed, and incubated with phycoerythrin-conjugated goat anti-rat IgG. 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 from $3x10^6$ mononuclear cells from peripheral blood of a healthy blood donor. 5 μg total RNA was reverse transcribed with Superscript II RT 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'-TTACCCTGACTTAGAG TCCAC-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 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. Following A-addition it was cloned into a T/A vector. The identity of insert was verified by sequencing.

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. In brief, inserts were produced by tagged random primed elongation and amplification using SpeI and NotI tagged random primers and CS cDNA as template excised with BamHI and EcoRI digestion from the plasmid mentioned above. Following purification and size selection inserts were digested with SpeI and NotI. 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 in a final volume of 5μl and incubated 48 hours at 4C . The ligation mixture was phenol-chloroform extracted, ethanol precipitated and packaged into lambda phage particles. Phage were amplified by infecting log phase E.coli BB4 cells and plating them on LB agar plates. After plaque formation phage were eluted, concentrated with precipitation and resuspended in buffer supplemented with protease inhibitors.

Affinity selection of CS antigen fragment library

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.

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. 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 the 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). 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.

We developed a set of mAbs against the Hepatitis B virus X antigen (HBX) for research and diagnostic use. Hepatitis B virus (HBV) is an important etiologic agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. 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. 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. 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.

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. This sequence can be found in the primary structure of HBX (amino acids 88.-93.).

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. This calculation has verified the results of the random peptide library screen.

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. 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.
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.

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.
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. Since previous studies have suggested that natural antibodies play an important role in the innate like component of the humoral immune response, 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. 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. To exclude the possibility of CS contamination in the nucleosome antigen preparation, we used our anti-citrate synthase mAb 4H3E5 in the same ELISA system. For further verification of our results we carried out competition ELISA experiments using CS from porcine heart as competitor.
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. 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. Our results show that there is indeed a cross-reactivity of anti-CS antibodies from SLE patients with nucleosome antigen.

CS antigen fragment library construction

Bacteriophage surface display of peptides is an extensively used technique for a variety of applications. The most commonly used systems are based on fusion to a filamentous phage coat protein. However, the life cycle of these phages limits the size of the displayed peptide, therefore we have chosen phage lambda for construction of a phage displayed CS antigen fragment library.

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. 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.

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. 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.

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. 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. 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. 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.

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 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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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

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