PhD Thesis

Applied bioinformatics in molecular biology and immunology

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Abstract:

Bioinformatics or **computational biology** is the use of techniques from applied mathematics, informatics, statistics, and computer science to solve biological problems. It is one of the most dynamically researched fields within science. It is one of the youngest among sciences. It has changed from biostatistics and molecular modeling to one of the most dynamically evolving science in the last half century. It connects biology with informatics. Thanks to the modern computer performance and development in algorithms *in silico* research become a good alternative to *in vitro* research.

The focus of this thesis is bioinformatics, program development, the use of bioinformatic tools and biostatistics.

The work included software development to help the lab work in large scale DNA sequencing projects. This resulted in the `swriter` program which eases the loading of gels from microtitter plates with Hamilton pipette. A software system a collection of scripts and programs were written to help data assessment in large scale DNA sequencing projects.

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.

We also used the HbxAg model to compare the epitope specificity, determined with phag random display libraries, with patient sera in different immunological states (active hepatitis, chronic hepatitis and symptomless carriers) and our MAb to our *in silico* predictions.

In the thesis a novel use of biostatistical methods is presented to clarify the hidden correlations among different parameters and groups within biological data. Correlation between the HBx serum concentration and anti-HBx antibody titers was analyzed by regression analysis enables to clarify the hidden correlations among different parameters and groups, which may have prognostic consequences.

Introduction

Bioinformatics or **computational biology** is the use of techniques from applied mathematics, informatics, statistics, and computer science to solve biological problems. It is one of the most dynamically researched fields within science.

Research in computational biology often overlaps with systems biology. Major research efforts in the field include sequence alignment, gene finding, genome assembly, protein structure alignment, protein structure prediction, prediction of gene expression and protein-protein interactions, the modeling of evolution and biostatistics.

The terms *bioinformatics* and *computational biology* are often used interchangeably, although the latter typically focuses on algorithm development and specific computational methods. (In the biology-mathematics-computer science triad, bioinformatics will intimately involve all three components while computational biology will focus on biology and mathematics.) A common thread in projects in bioinformatics and computational biology is the use of mathematical tools to extract useful information from noisy data produced by high-throughput biological techniques. (The field of data mining overlaps with computational biology in this regard.) Representative problems in computational biology include epitope prediction, and the prediction of gene regulation with data from mRNA microarrays or mass spectrometry.

The results of modern molecular biology and immunology with the algorithm and computational power increase in the 20th Century, made bioinformatics one of the most dynamically developing field of research.

The efficient fine molecular mapping techniques are requiring the solution of specific (bio)informatical problems for the better understanding of the relation between the bimolecular' structure and functions. 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.

Various algorithms are available for calculation of immunogenity index and predicting epitopes on antigens for further immunological studies. 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 pola,m ,r 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.

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 micro organisms, the hazard of bio-terrorism, or bio-therapies for cancer, organ transplantation as well as autoimmune diseases. By predicting epitope one would be able to develop specific monoclonal antibodies with short peptide immunization raising antibodies cheaper and quicker.

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. Laboratory diagnostics, drug design and vaccine developments are intensively growing areas of current medical biotechnology where computing techniques play an essential role. However, the evidence based verification of these

bioinformatically predicted epitopes targeted by autoantibodies has been the focus of recent studies only.

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.

Epitop prediction:

The study of **immunological recognition**, its modifications by molecular interactions and the physico-chemistry of antigens is the mainstream of today's immunological research. The 3D structures of hydrophilic/hydrophobic and electrostatic interactions an antigen play an important role in immunological recognition and response. The surface characteristics of a given molecule is determined primarily by its amino acid sequence.

It was the development of MAb techniques, what made possible the detailed characterization of epitope-paratope interactions through well defined model experiments. The data collected by these experiments clarified our knowledge about epitope recognition.

Epitope or **antigen determinant** is that part of a molecule what is recognized by antibodies. Epitopes are relative entities, because complementary paratopes are needed for their recognition. Epitopes have antigenic reactivity, they are able to bind to antibodies, and they are able to induce immunresponse (antiidiotypic response).

Protein surfaces may be regarded as a network of overlapping epitopes. Among these epitopes there are immundominant and less reactive epitopes. The number of epitopes on any given surface is defined by the number of MAbs which may be produced against the protein. X-ray crystallography localized the epitope-paratope binding to an area of 20Å x 30Å on the molecular surface. It is unlikely that this epitopes are composed of amino acids following each other – **linear epitopes**. It is much more likely that these epitopes are **conformational epitopes**.

From an immunological point, both linear and conformational epitopes are important. From the immunbiotechnological point linear epitopes are more important.

It is only the X-ray crystallography and NMR which determine the exact epitop-paratope binding. Other methods (e.g.: mass spectroscopy, overlapping peptide libraries) give only an estimation of the epitope-paratop binding.

The rapid development of informatics and our knowledge of epitope-paratop binding made it possible to predict epitopes from the primary sequence of a molecule. These algorithms predict the tertiary structure or other attributes (secondary structure, mobility, hydrofobicity, hydrofilicity etc.) of a possible epitopes from the primary structure (amino acid composition) of a sequence.

All prediction methods work along the same principles. A table is constructed, a value describing a given attribute of an amino acid as a possible epitop (secondary structure, mobility, hydrofobicity, hydrofilicity, acrophylity etc.) is assigned to each of the 20 amino acids. When making a prediction profile, these values are summed in a 5-21 amino acid long window which is moved along the examined sequence. The results are plotted and presented in table (e.g.: in case of a hydrophilicity profile, the higher values correspond to more hydrophilic regions). To avoid extremes in the profile, the next step is refining the results. There are two widely accepted methods to do this: *Karplus* and *Schulcz* weight the positions in the window; van Regenmortel uses a Gaussian distribution. Prior to predicting epitopes, the profile is normalized.

Most methods predict epitopes from the primary sequence through the analysis of secondary structure or some attributes of the composing amino acids. These examined attributes may include the hydrofobicity, hydrofilicity, acrophility, mobility, atomc mobility, α-helix and β-sheet structure probability of the composing amino acids. Several tools were developed for the use of these

methods. Simpler software such as the HYDRO use one method, while the more advanced tools (e.g.: *Wisconsin GCG Package*, *PrediTop*) use several of them.

The amino acid composition, hydrogen bonds, the side-chain interaction define the secondary structure of proteins, the α-helix and β-sheet regions. Approximately 50% of a protein is located at turns, while 50% in repeated structures (25 % in α-helix and 25% in β-sheet). The secondary structure determines the antigenity of a protein.

The α-helix and β-sheet are hydrophobic regions, usually localized inside the molecule, while the flexible turns and curves are positioned on the hydrofil surface of the molecule. These presumptions are used in the *Chou-Fasman* and *Garnier* algorithms which has a detection rate of 55-70% of epitopes.

Hopp and *Wood* algorithms presumes that hydrofilic regions are located on the surface of a molecule. The *hydropathic index of Kyte and Dolittle* assumes the water solubility of side chains, *Parker, Guo and Hodges* examined the hydrofilicity of amino acids with HPLC. *Hopp's akrophylicity index* was created by studying the surface exposition of amino acids on 49 proteins with known structure. The *Karplus and Shultz flexibility index* correlates flexibility with surface probability. *Welling* studied the amino acid composition of 606 epitops on 20 proteins. In his study, all positions within an epitope were weighted evenly. This is to be considered, using his method, since there are significant positions in an epitope, which has a dramatic effect on immunogenity.

The studies of *Jameson and Wolf* proved that combining different methods will improve epitope prediction. In their studies 5 different algorithms were used simultaneously, increasing specificity, without decrease in sensitivity.

Effectiveness of a method is depending much on the used algorithm, the examined protein, the circumstances (protein, immunological state etc.) used for fine tuning the method. The specificity and sensitivity in increased if we use an algorithm, trained similar way to the circumstances it is used for predicting epitopes.

Epitope prediction had become a self-standing branch of bioinformatics. With the *in silico* prediction of epitopes there are new possibilities in research. The time needed to produce new antibodies is shortened, the laboratory work is less (one may immunize with a given, specific sequence only). It is possible to develop new vaccines against microorganisms and diseases (e.g.: hepatitis, HIV, influenza, caries).

In silico research opened new perspectives in immunobiology. However without the proper knowledge of the limitations the results may be interpreted falsely.

The further development, improvement of novel and existing algorithms and tools are necessary, on well characterized large samples to improve our understanding of epitop-paratop binding.

Aims

My aim was to develop new and improve existing bioinformatic tools and methods applicable in molecular biology and immunological research, as well as their critical review. I was concentrating on 4 areas:

1. Software development to help the lab work in large scale DNA sequencing projects.
2. Software development to help data assessment in large scale DNA sequencing projects.
3. The *in vitro* immunoserologiacal validation of *in silico* predicted epitopes.
4. The combined use of biostatistical methods to assess the connection among biological data.

Materials and methods

Bioinformatic tools helping large scale DNA sequencing

cDNA library, template preparation and sequencing: The cDNA library was constructed with oligod(T)-primed *T. cruzi* CL Brener epimastigote polyA+ RNA, by directional cloning into a pT7t318D phagemid vector with a modified polylinker. The library was subsequently normalized to reduce the representation of abundant mRNA species. The cDNA library was transformed into DH5-alpha strain of *E. coli* and more than 23 000 individual colonies were randomly picked and ordered into 384-well microtiter plates.

Automated fluorescent cycle sequencing reactions were performed using dye-primer cycle sequencing chemistry. The ABI Prism™ -21M13 fluorescent dye-labeled primer kit (and DYEnamic direct cycle sequencing using T7 dye-labeled primer, were used as described by the suppliers. In both cases, a Beckman Biomek 2000 robotic workstation was used for aliquoting and mixing of the reagents. Cycling reactions were run in a GeneAmp 9600 thermal cycler or a PTC-225 Peltier thermal cycler (MJ Research), followed by ethanol precipitation of the sequencing reaction products as described. The samples were analyzed on ABI 373 or ABI Prism 377XL DNA sequencers (Applied Biosystems) according to the protocol.

Sequence analyzing system: For sequencing an ABI Prism ® 377XL was used. The raw data was analyzed on an Apple Macintosh Power PC with ABI Prism® DataCollection Software.

Programming and compiling: Scripts were written in `bash`. Programming was done in C++ and complied with GNU GCC.

Epitope mapping

In silico epitope prediction

B-cell epitope prediction:

Mitocondrial CS epitope prediction: The human mitochondrial CS sequence (EC 2.3.3.1; Gene Bank accession no. **PID O75390**) used for prediction analysis was obtained from the protein sequence database.

To localize the potentially antigenic sites, we have predicted β-turns of CS protein segments using the *Chou-Fasman* secondary structure prediction method and hydrophobicity predictions by Eisenberg. 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 ($P_{hydrophobicity} < 0$) were selected. We also used the *PepPlot*, *PeptideStructure* and *PlotStructure* programs of the *GCG Wisconsin Package* for surface probability ($P_{surface} > 1$), Jameson-Wolf antigenity index ($P_{JW\ antigenity} > 1$) (Jameson and Wolf, 1988) and hydrophilic regions ($P_{HW\ hidrophilicity} > 1$) to fine tune our prediction results.

Swiss-Model Automated Protein Modeling Server 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.

Hepatitis B-virus X-protein epitope prediction: The *Chou-Fasman* and *Garnier-Ousguthorpe-Robson* secondary structure prediction algorithms ($P_{hydrophobicity} < 0$; $P_{\beta\text{-turn}} > 1$) and the *Jameson-Wolf* antigenicity index ($P_{JW\ antigenity} > 1$) was used for HBX epitop prediction in the *Wisconsin GCG Packag ProteinStructure* program.

T-cell epitope prediction: T-sejt epitóp térképezés: 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 *TEPITOPE2000* program. 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.

In vivo validation of epitope prediction

Recombinant HBxAg overlapping fragments and synthetic peptide fragments were used for the development of the assay.

Examined samples:

a.) CS: 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. The age and sex ratios of patient groups were matched.

b.) HBX: Sera of patients suffering from 14 HBV positive acute and 80 chronic hepatitis and those taken from 12 healthy HBV carriers were compared with 22 healthy - HBV negative - serum samples. The sera were pre-tested for HBV positivity by HBsAg and HBcAg specific ELISA (Dialab, Hungary).

Immunoserological detection of anti-CS autoantibodies with indirect ELISAl: Indirect ELISA was used for immunoserological detection of anti-CS autoantibodies. 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.

Epitop mapping with pin-attached ELISA: Forty decapeptides overlapping five amino acid residues were synthesized, corresponding to the nine regions predicted, on β-alanyl-glycine functionalized polyethylene pins with Fmoc/tBu chemistry according to *Geysen*'s method. 96-well polystyrene ELISA plates and pin-bound peptides were the hardware of epitope mapping. The color reaction was developed with *o*-phenylenediamine and measured on iEMS MF micro photometer at 492 nm (OD). The data were analyzed with Ascent Software V.2.4.2.

CS 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 = 20000; Matrix PAM30; Gap cost Existence: 9 Extension: 1; Word size = 2, database size as of September 2004 = 688 443 072). Immumologically relevant hits showed homology with proven or hypothetical CS, but only negligible similarity was found between CS and non-CS proteins.

Screening of random peptid 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 constructe. The affinity selection of phages with our anti-HBX Mab was performed using the biopanning technique. Forty positive clones were selected and tested further with ELISA. According to the results of ELISA 10 clones were selected for sequencing.

Combined application of biostatistical methods:

Statistical analysis of the collected data was done using on an IBM PC, running SPSS for Windows, release 9.0. The regression analysis of ELISA results in all 3 groups (acut, chronic and carrier) were performed against all samples in a matrix (N, C, C1, C2, C3, X, $X^{14\text{-}16}$). Pairs with an absolute regression value of less than 0.4 ($|R| > 0.4$) and/or P value was larger than 0.05 ($P > 0.05$) were rejected. Results were presented in a table.

Results

Automated Sequence Annotator (*AUSA*)

With our scripts and programs and applications available freely we developed an automated system. This system a collection of programs and scripts was capable of without human intervention transfer the chromatograms from an Apple computer to Unix platform, and then screens, quality assess and edits the sequences. In the next step, the sequences are homology searched to local and public databases, finally annotated and organized in an e-mail form to be submitted to databanks after manual editing and confirmation of the results.

Our script is called *AUSA* (*AUtomated Sequence Annotator*) is run as a `cron job` in given intervals automatically. The *AUSA* scripts move the chromatograms from a given directory, transfer it from the sequencer connected Apple Power PC to a UNIX/Linux machine through *netatalk* protocol. Here the script performs automatic backup and invokes the *phred* base-caller. *Phred* base calls the sequences from the original chromatograms (`.chr` extension) and gives a sequence (`.seq`) and a quality file describing the quality of the given sequence (`.qual`). In the next step *ACE* (*Automated CDNA Editor*) is called.

Automated CDNA Editor (*ACE*)

The *ACE* program, developed by us, quality cuts and edits a given sequence (`.seq` file) according the quality of sequencing (`.qual` file), and command line input or config file (`.aconf` file).

In the first step the program performs a local alignment according the original *Smith-Waterman* algorithm with freely given primers (gap-penalty, extension score, match score). Due to the short sequences (limited by sequencing performance to max. ~500 bases) running time is fast even on Intel Pentium MMX 166MHz.

In the second step, low quality regions are removed. In case of poliA tails these sequences are removed too.

The output of *ACE* program is a clipped sequence and an integer. The later determines if all steps could be performed, or there was an error and the program was terminated with an error. This integer is used later by the invoking script to decide the future of the given sequence (good, bad, needs manual checking).

The next step is low complexity screening with the *dust* and *seq* programs. The *blastcli* client runs BLASTN and BLASTX programs, thus performs homology searches.

Results are tabulated with *btab* program. Results are analyzed by our *ctab* program.

Next the *MAKEMAIL* program is invoked.

MAKEMAIL

The *MAKEMAIL* program makes a file in NCBI batch submission for to be submitted to GENBANK via e-mail. The input of the program is a configuration file, or command line options, where data necessary for submission (project name, references, authors etc.) are given. The program receives the sequence data from the edited fasta format sequences (`.fasta` file, ACE edited sequence) and the *ctab/btab* format search result files.

The user may check the output of *MAKEMAIL* prior submission. This is strongly recommended even in the case of strict *ACE* and *BLAST* parameters. The low quality and short sequences may be checked manually.

SWriter (Sheet Writer)

The program helps to track samples after loading 96-well microtiter plates on 64 lane gels with 8 syringes Hamilton pipettes. The configuration of the Hamilton pipettes will load every second lane on a gel, thus one may easily lose track of samples.

The program *SWriter* runs on Unix platform. *SWriter* takes manually given sample names or the nomenclature used in the laboratory of 2 96 well microtiter plates and writes a 3 48 lane gelfiles.

The use of *SWriter* makes gel naming, loading and sample tracking easier, and avoids some of the common mistakes mixing samples on gels.

Epitope maping

In vitro validation of *in silico* predicted epitops

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.

CS 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.

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. 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 to IX were prepared by solid phase chemistry.

CS 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. 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).

In vitro Results of ELISA:

Sera having an OD value at least two standard deviation (2SD) 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**.** Serum samples found positive during the simple binding ELISA test were used for the further epitope mapping.

Immunoserological epitope mapping in multipin system:

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. The results of detailed immunopathological analysis on the differences between the results of the autoimmune and healthy samples are 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 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 6 peptides which were not predicted but synthesized, 3 were not and 3 were recognized by autoantibodies. One unpredicted synthetic peptide (330-339 amino acid sequence) had strong reactivity with the different groups of sera.

The results of the homology searches of the *in silico* predicted and *in vitro* analyzed epitopes are presented in. 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.

Random peptide library screening with CS purified sera:

With random peptide library screening we got 2 positive sera of autoimmune patients, in the region 145-150 amino acids. This correlated well with our *in silico* predictions, and overlapped with the predicted 138-161 region. Immunization with CS resulted Mab with a specificity (31-59 amino acids) this showed partial correlation with the predicted 51-78 amino acid region.

HBxAg B-cell epitop prediction results:

The exact 3D structure of HBxAg is unknown. The protein contains 84 hydrofob amino acids, which is 54% of all amino acids in HBxAg. The *Cho-Fasman* and *Garnier-Osbourne-Robson* algorithms predicted turns at the N- and near the C-terminals of the molecule. Between 75-130 amino acids an α-helix is predicted. Secondary β-sheet is highly probable all along the whole sequence. Glicolization sites are unknown.

The *Jamson és Wolf* antigenicity index predicted sequences 22-31 and 100-114 as probable epitopes. In vitro validation of the prediction was done with M13 filamentous phage expressed random peptide library. Our Mab recognized the 88-93 amino acid of HBxAg. This results did not correlate with the previous predictions.

In vitro ELISA results:

The HBxAg serum levels were measured by sandwich type ELISA. The absolute concentrations were scattered in a wide range between 18 – 1800 ng/ml. The averages of the serum HBxAg concentrations were higher in both acute and chronic hepatitis samples, than in symptomless carriers, however, the individual variations were also the highest in chronic hepatitis patients.

The individual differences were relatively homogenous except for chronic hepatitis patients with anti-HBx IgG isotype. The strongest anti-HBx reactivity was found on the longest fragment of the

recombinant antigens (10-143) with both IgG and IgM isotypes. The first recombinant C terminal fragment (C1: 79-117) was characteristically positive in chronic hepatitis patients with both IgG and IgM isotypes. The IgG antibodies were strongly positive on the same sequence in acute hepatitis and in symptomless carriers. The anti-HBx IgM positivity was not significantly stronger on C1 sequence in any of the group examined. The other investigated sequences were slightly positive with minimal individual variations. Healthy controls proved to be consequently non-reactive on all antigens included into the study.

Correlation between the HBx serum concentration and anti-HBx antibody titers was analyzed by regression analysis enables to clarify the hidden correlations among different parameters and groups. Strong and homogenous correlations were found in ***acute hepatitis*** for the all measured HBxAg fragments in IgM response. The IgG antibodies showed correlation between the high recognition of C1 fragments and consequent ignorance of the N terminal end. The strong correlation between the serum HBxAg level and the antibody production against the longest fragment (X: 10-143) shows good probability of the mathematical analysis.

Results of combined biostatistical analysis

Correlation between the HBx serum concentration and anti-HBx antibody titers was analyzed by regression analysis enables to clarify the hidden correlations among different parameters and groups. Strong and homogenous correlations were found in ***acute hepatitis*** for the all measured HBxAg fragments in IgM response. The IgG antibodies showed correlation between the high recognition of C1 fragments and consequent ignorance of the N terminal end. The strong correlation between the serum HBxAg level and the antibody production against the longest fragment (X: 10-143) shows good probability of the mathematical analysis.

In ***chronic hepatitis*** B patients we found IgG dominance and correlations among C terminal fragments but no correlation with N terminal fragments. The serum level of HBxAg moderately influenced the correlations in the IgG response against the C terminal fragments and the longest fragment (X: 10-143). The regression analysis of IgM reactivity with N terminal and C terminal fragments were dominant and no correlation was found with the antigen concentrations in the same sera.

The regression analysis of the results of ***symptomless HBV carriers*** showed close similarities to acute hepatitis B: the IgM dominance was typical and there was a strong correlation in the IgG response between the C1 and N terminal fragments. However, the IgM dominance was less homogenous in comparison to the patients suffering from acute hepatitis, and further IgG related correlations were detected between the HBxAg concentration and the N-terminal fragment-specific IgG production probably indicating, that HBxAg immuncomplexes are composed of IgG and antigen.

Discussion

In silico experiments became the accepted methodology in molecular biology and immunology. The use of statistical methods had changed natural sciences. The advances in algorithm development, computation and information technology made the handling of large databases possible. The spreading of personal computers, modern programming languages widened the place for computer aided research. The breakthrough of modern molecular biological techniques (e.g.: sequencing, microchips, etc.) not only increased the amount of biological data, but also increased the need for its assessment. A new science bioinformatics had evolved.

Major research in the field include sequence alignment, gene finding, genome assembly, protein structure alignment, protein structure prediction, prediction of gene expression and protein-protein interactions, the modeling of evolution and biostatistics.

The work included **software development** to help the laboratory work in large scale DNA sequencing projects. This resulted in the `swriter` program which eases the loading of gels from microtitter plates with Hamilton pipette.

A software system a collection of scripts and programs were written to help data assessment in large scale DNA sequencing projects under Linux. This package full fields the needs of our laboratory work.

We have used a well-conserved citrate synthase and HBxAg models to validate *in silico* predicted epitopes with *in vitro* immunoserologically verified epitopes of circulating autoantibodies.

The *in silico* analysis coincided frequently, but not always, with the immunoserologically determined epitopes. 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. 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. 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.

In the thesis a novel use of **biostatistical** methods is presented to clarify the hidden correlations among a high number of different parameters and groups within biological data. Correlation between the HBx serum concentration and anti-HBx antibody titers was analyzed by regression analysis enables to clarify the hidden correlations among different parameters and groups, which may have prognostic consequences.

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Publications

Publications related to the thesis:

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1. Czömpöly T, Olasz K, Simon D, **Nyárády Z**, Pálinkás L, Berki T, Németh P: *A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network?* Molecular Immunology (accepted, MIMM-D-05-00212) **IF 2.827**

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31. Németh P, Nagy G, Czömpöly T, **Nyárády Z**: *Mikroanalitikai és molekuláris biológiai lehetőségek az ellenanyagok és T sejtek epitop specifitásának feltérképezésében (Microanalitical and molecular biological possibilities of antibody and T cell epitop specificity mapping)* XXth Conference of Hungarian Immonological and Clinical Immunological Associations, May 9-10, 2003, Salgóbánya, Hungary
32. Gelencsér G, **Nyárády Z:** *Szájüregi daganatos betegek korszerű fájdalomcsillapítása (Up-to- date pain releife of patients with oral cancer).* Xth Pannon Section of the Association of Hungarian Oral and Maxillofacial Surgeons, November 23, 2002, Pécs, Hungary
33. **Nyárády Z,** Olasz L**,** Németh Á, Gelencsér G: *Platysma lebeny alkalmazása nagy kiterjedésű defektusok zárására (The use of platysma transpositional flapin the reconstruction of massive through-and-through defects).* Xth Pannon Section of the Association of Hungarian Oral and Maxillofacial Surgeons, November 23, 2002, Pécs, Hungary
34. **Nyárády Z,** Pintér A, Olasz L, Vastyán A, Lovász M, Kopcsányi G, Vincze O, Kárpáthy M, Eperjesi B: *Ajak és szájpadhasadékos gyermekek csontpotlásábval szerzett tapasztalatink: A Pécsi Hasadék Munkacsoport előadása. (Our experinece with bone grafting of cleft-lip and –palate patients: A paper of the Pécs Cleft Team.)* 6th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 10-12, 2002, Szombathely, Hungary (Hungarian)
35. Rónai A, **Nyárády Z,** Gelencsér G, Olasz L: *Malignus lymphoma primer orális manifestatiojanak ritka esete: Esetbemutatás (A rare case of primary oral manifestation of malignant lymphom: A case report).* 6th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 10-12, 2002, Szombathely, Hungary (Hungarian)

36. Olasz L, **Nyárády Z,** Gelencsér G, Rónai A: *Oro-pharyngeális daganatok kombinált kezelésének lehetőségei és előnyei. (The possibilityes and advantages of combined therapy of oro-pharyngeal cancers.)* 6th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 10-12, 2002, Szombathely, Hungary (Hungarian)
37. **Nyárády Z,** Mörnstad H*: Mathematical functions for age estimation of Hungarian children using the modified Demirjian method.* HDA Árkövy – FDI World Dental Congress 2002, 1-5 October, 2002, Vienna, Austria
38. Olasz L, **Nyárády Z,** Németh Á, Rónai A, Gelencsér G: *Regional failure related to alkylating agents in neoadjuvant chemotherapy of oropharyngeal planocellular cancer.* XVIth Congress of the European Association for Cranio-Maxillofacial Surgery, 3-7 September 2002, Münster, Germany
39. **Nyárády Z,** Olasz L, Németh Á: *Reconstruction of massive through-and-through facial defects using platysma transpositional flap.* XVIth Congress of the European Association for Cranio-Maxillofacial Surgery, 3-7 September 2002, Münster, Germany
40. Rónai A, **Nyárády Z,** Gelencsér G, Olasz L: *A rare case of primary oral manifestation of malignant lymphoma.* XVIth Congress of the European Association for Cranio-Maxillofacial Surgery, 3-7 September 2002, Münster, Germany
41. Olasz L, Gelencsér G, **Nyárády Z,** Rónai A: *Metastatic failure related alkylating agents in induction chemotherapy of oropharyngeal planocellular cancer.* 4th Danubius Conference on Oral and Maxillofacial Surgery, April 24-27, 2002, Rovinj, Croatia
42. Rónai A, Olasz L, Gelencsér G, **Nyárády Z***: Rare case of primary oral manifestation of malignant lymphoma.* 4th Danubius Conference on Oral and Maxillofacial Surgery, April 24-27, 2002, Rovinj, Croatia (Poster)
43. Németh Á, **Nyárády Z**: *Daganatok molekuláris epidemiológiája (Molecular epidemiology of tumors in head-and-neck region.)* „Mandulavirágzási Napok" March 4-8, 2002, Pécs—Szekszárd, Hungary (Hungarian)
44. Olasz L, Gelencsér G, **Nyárády Z,** Rónai A: *A kombinált terápia előnye előrehaladott carcinómás betegek ellátásában. (Advanteges of combined chemotherapy protocolls in the treatment of advanced carcinomas.)* 5th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 30-December 1, 2001, Budapest, Hungary (Hungarian)
45. Olasz L, **Nyárády Z,** Pintér A, Vástyán A, Lovász M, Kopcsányi G, Vincze O, Kárpáthy M, Eperjesi B: *A Pécsi Hasadék munkacsoport klinikai gyakorlata. (Clinical practice of Pécs Cleft Lip and Palate Team.) Video presentation* 5th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 30-December 1, 2001, Budapest, Hungary (Hungarian)
46. **Nyárády Z,** Olasz L, Rónai A, Gelencsér G: *Alternatív megoldás az intraoperatív okklúzió biztosítására. (An alternative way to secure intraoperative occlusion)* 5th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 30-December 1, 2001, Budapest, Hungary (Hungarian, Poster)
47. Rónai A, Olasz L, **Nyárády Z,** Gelencsér G: *Impactált és számfeletti fog együttes előfordulásának ritka esete. (A rare case of impacted and supernumeric tooth)* 5th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, November 30-December 1, 2001, Budapest, Hungary (Hungarian, Poster)
48. **Nyárády Z,** Pintér A, Olasz L, Vastyán A, Lovász M, Kopcsányi G, Vincze O, Kárpáthy M, Eperjesi B: *Clinical practice of Pécs Cleft Lip and Palate Team. Video presentation.* The 9th International Congress on Cleft Palate and Releated Anomalies, June 25-29, 2001, Göteborg, Sweden
49. **Nyárády Z,** Pásztor E, Olasz L, Szabó Gy*: Submental endotracheal intubation in concurrent orthognatic surgery.* The 9th International Congress on Cleft Palate and Releated Anomalies, June 25-29, 2001, Göteborg, Sweden (Poster)

50. Németh Á, Gyöngyi Z, Olasz L, **Nyárády Z**, Ember Á, Ember I: *The effect of cisplatin on early oncogene activation in vitro.* 2001, Greece (Poster)
51. Pintér A., Vástyán A, Vincze O, Lovász M, Kárpáthy M, Eperjesi B, Kopcsányi G, Olasz L, **Nyárády Z**: *The complex treatment of cleft lip and palate patients and their follow up*. "Zala" Symposium on the Actual Questions of Pediatrics. May 16, 2001, Zalaegerszeg, Hungary (Hungarian)
52. **Nyárády Z**, Németh Á: *Effects of cisplatin-containing chemotherapy protocol on the molecular epidemiology of head-and-neck cancers.* FEBI Symposium of Young Oncologists, May 11, 2001, Pécs, Hungary (Hungarian)
53. Olasz L, **Nyárády Z,** Szentirmay M*: Assessment of relieving symptoms of xerostomia with oral pilocarpine during irradiation in head-and-neck cancer patients.* 5th International Symposium of Predictive Oncology and Therapy, October 28-31, 2000, Geneva, Switzerland
54. Olasz L, **Nyárády Z**, Németh Á, Tornócky T*: A randomized study of cisplatin-containing combined chemotherapy protocol.* 5th International Symposium of Predictive Oncology and Therapy, October 28-31, 2000, Geneva, Switzerland
55. **Nyárády Z**, Olasz L, Németh Á, Tornóczky T: *A randomized study of cisplatin-containing combined chemotherapy in head and neck planocellular cancers.* 4th National Congress of the Association of Hungarian Oral and Maxillofacial Surgeons, October 26-28, 2000, Debrecen, Hungary (Hungarian)
56. Németh Á, Ember I, **Nyárády Z,** Olasz L: *Molecular and predictive epidemiology of head and neck tumors.* 4th national Congress of the Association of Hungarian Oral and Maxillofacial Suregons, October 26-28, 2000, Debrecen, Hungary (Hungarian)
57. Szántó I, **Nyárády Z**, Szabó Gy, Solti P: *Prosthetic rehabilitation of an advanced sclerodermic patient.* 13th Congress of Hungarian Prosthetic Association, September 17-19, 1999, Pécs, Hungary (Hungarian - poster)
58. Olasz L, Németh Á, **Nyárády Z,** Tornóczky T: *A randomized clinico-pathological study of cisplatin (preliminary results).* 14th Congress of Hungarian Chemotherapy Association, Jun 2-4, 1999, Pécs, Hungary (Hungarian)
59. **Nyárády Z**, Mörnstad H: *Dental development of Hungarian Children*. UGSBR Conference, Feb, 1997, Uppsala, Sweden