Immuno-biotechnological model-experiments on

Hepatitis B virus X antigen

a protein with unconventional physico-chemical properties

PhD-thesis

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Introduction

Hepatitis B virus (HBV) is a major pathogen that chronically infects more than 400 million individuals worldwide. HBV is known as an important cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). HCC is one of the most common cancers worldwide, with 250,000 new cases diagnosed each year. Based on epidemiologic studies involving chronic HBV infection, it is estimated that the relative risk of developing HCC for HBV carriers may be 100- to 200-fold higher than that for non-carriers. 500,000 lethal consequences of HBV-related chronic hepatitis or HCC are registered. Perinatal transmission results in lifelong symptomless but infectious carrier status with great epidemiological importance. The probability of perinatal transmissions has been found to be about 55% in the case of first-born babies, and about 40% in the case of later pregnancies of symptomless carrier mothers.

HBV is a small and partially double-stranded circular DNA virus belonging to the hepadnavirus family that includes hepatitis viruses of woodchuck, ground squirrel, Peking duck, *etc*. HBV genome has four open reading frames (ORFs), including envelope genes coding region (*pre-s1*, *pre-s2* and *s* gene coding region), precore (*pc*) gene and core (*c*) gene coding region, polymerase (*p*) gene coding region, X gene coding region. X gene is the smallest and highly conserved open reading frame of HBV. X gene codes for a 17-kda protein (X protein, HBxAg), which is made of 154 amino acids. HBxAg, which has no homologue in the host genome. The X gene is conserved among mammalian hepadnaviruses. HBxAg is a multifunctional viral regulator that modulates transcription, signaling pathways, protein degradation, and cell responses to genotoxic stress and signaling pathways.. These modulations affect viral replication and viral proliferation, directly or indirectly. HBxAg also affects cell cycle checkpoints, cell death, and carcinogenesis. Importantly, HBxAg is a moderate but broad-acting transcriptional transactivator and activates a variety of cellular and viral genes, including proto-oncogenes, such as c-myc, c-fos, and c-jun. However, little is known about the exact role of HBxAg in tumorigenesis. The X proteins of HBV can moderately stimulate transcription of many different viral and cellular transcription elements AP-1, CREB, NF-B and C/EBP. Promoters and enhancers stimulated by HBx typically contain DNA binding sites for NF-_B, AP-1, AP-2, c-EBP, ATF/CREB, or the calcium-activated factor NF-AT. Biochemical evidence for activation of the DNA binding activity of NF-B, AP-1, ATF/CREB, and NF-AT. HBxAg activates cell signaling cascades involving mitogen-activated protein kinase (MAPK) and Janus family tyrosine kinase (JAK) signal transducer and activators of transcription (STAT) pathways.

Aims of the thesis

1. Mapping of HBxAg immunogenity on computer models and developement of recombinant HBxAg fragments based on predicted data.
2. Development of anti-HBxAg monoclonal antibodies against recombinant and synthetic antigens. Characterization of antibodies *via* immuno-serology and immuno-histochemistry.
3. Determination of epitope specificity of monoclonal antibodies produced against recombinant HBxAg antigen
4. Immuno-histochemical studies on formalin fixed and paraffin embedded slides, originated from human liver biopsy.

5. Development of an immunoassay (sandwich ELISA) to measure the X antigen of hepatitis B virus from serum. Detection of natural antibodies produced against the X antigen of hepatitis B virus using the developed recombinant HBxAg fragments.
6. Development of a second generation immuno-serological method available to study multiple parameters, by the use of micro-beads for quantitative measurement of circulatory HBxAg.

Materials and Methods

Antigenity prediction and synthetic peptides

The Chou–Fasman and the Garnier–Osguthorpe–Robson secondary structure predictions 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.

Antigens

Two construct of recombinant HBxAg were used (Marczinovits et al. 1997) for monoclonal antibody and assay developments. The hepatitis B virus X gene (HBx) was cloned into the fusion expression vector pRIT2T or into pGEX-3X, resulting a HBx-staphylococcal protein A (HBxAg-pA) or a HBx-GST (HBxAg-GST) fusion gene construct, respectively. Both constructs were expressed in *E.coli* DH5. HBxAg-pA was used for immunization in crude form. Affinity purified HBxAg-GST was used for testing the antibodies.

Recombinant X antigen (10-143. amino acid):

The development of the recombinant construct of HBxAg was described previously (Marczinovits et al., 1997). Briefly, the truncated X gene was obtained by cutting of the pHB320 plasmid with *BamH*I-*Fsp*I restriction enzymes and cloned via the pHSG 395 vector into *BamH*I-*Sma*I restriction sites of the fusion-expression vector pGEX-3X (Amersham Pharmacia Biotech), creating the pGEX-3XXBF recombinant plasmid. The X protein is truncated by nine and eleven amino acids at the N- and C-termini, respectively. According to the cloning strategy, the truncated X protein is flanked by five amino acids at the N-terminus and by four amino acids at the C-terminal, derived from the pHSG 395 and pGEX-3X plasmids, respectively.

N terminal fragment (10-90 amino acid) of HBxAg:

The original plasmid (Bichko et al., 1985) (pHB320) containing the HBV DNA subtype *ayw* was used to subclone the entire HBx coding region between the *Nco*I site (position 1370 on the HBV genome) and *Bgl*II site (position 1986) into the pGEX-3X vector (Amersham Pharmacia Biotech) producing the pGEX-3XX recombinant plasmid. Clones containing only the N-terminal portion of the HBx coding region were obtained by cutting the pGEX-3XX recombinant plasmid with *Ava*I (position 1461 on HBV genome) and *EcoR*I (linker sequence of the vector), blunt-ending with Klenow fragment of DNA polymerase I followed by ligation.

C terminal overlapping fragments:

The pGEX-3XX plasmid was used to produce the recombinant overlapping HBx fragment. Three overlapping parts of the X gene were amplified by PCR using primers with *BamH*I and *EcoR*I restriction sites we published previously (Pal et al., 2003). The fragments were cloned into the pGEX-6P-1 expression vector (Amersham Pharmacia Biotech). Each construct was verified by sequencing.

The recombinant plasmids were transformed into *E. coli* strain DH5α, induced with isopropyl-thio-B-D-galactopyranoside (IPTG) and the recombinant fusion proteins were purified on glutathione-S transferase resin according to Marczinovits et al., 1997.

Synthetic peptide antigens

Peptides of 13-26 amino acids were synthesized using fmoc chemistry and purified by HPLC using routine techniques at the Department of Chemistry, Faculty of Medicine, University of Szeged, Hungary.

Hybridoma development

Female BALB/c mice (Charles River Inc., USA) were immunized repeatedly. The splenocytes of the most responding animal were fused to Sp-2/0-Ag14 (ATTC, USA) mouse myeloma cells according to the method described by Köhler and Milstein (1975). Hybrids were selected on HAT containing culture medium (DMEM, Gibco, USA).

Development of the capture antibody

The affinity purified recombinant HBxAg-glutathione *S*-transferase (GST) construct was the first antigen and the hybridoma clones were characterized by various methods. A clone (3F6/G10) was chosen as the capture antibody. Selectivity was tested in human liver biopsy samples from a retrospective immunohistochemical study.

Development of the detecting monoclonal antibody

Detecting antibody was developed against synthetic peptide fragments (13–26) of HBx. The 13–26 peptide antigen was conjugated to KLH carrier molecules. Balb/c mice (Charles River Laboratories, Raleigh, USA) were sequentially immunized. Hybridomas were produced (Köhler and Milstein, 1975; Najbauer et al., 1986) and cultured in DMEM (Sigma Chemical Co., USA) containing 10% FCS (Gibco, USA). The purified monoclonal antibodies were labeled by biotin (Sigma, USA). The best clone (4F1/A9) was selected for equally highest affinity to the synthetic antigen, to the recombinant HBxAg, and to human serum samples.

Immunoserological characterization by ELISA

Cell culture supernatants were first tested by simple-binding ELISA described by Engvall et Perlman (1972). HBxAg-GST was used as antigen for screening to exclude cross-reactions with the carrier proteins used for immunization.

Microtitre plates (Dynatech, USA) were sensitized with 5μg/ml of recombinant HBxAg in 0.1M bicarbonate buffer, at pH 9,6 overnight at 4°C, PBS-gelatin (0,5%) (Sigma, USA) was used to saturate the free binding sites. The hybridoma supernatants were tested in different dilutions. Reactions were developed by HRPO labeled rabbit anti-mouse Ig (Dakopats, Denmark) and measured by microphotometer (Dynatech MR 7000, USA) at 490 nm wavelength.

The isotype subclasses of the mAbs were determined using a mouse isotype kit (Sigma Chem. Co., USA)

Immunoblot determination

The HBxAg-GST and HBxAg-pA fusion proteins and the glutathione S-transferase (GST) enzyme protein were used for SDS-PAGE electrophoresis (Laemmli, 1970). The samples were run on a 15% polyacrylamide gel and stained with Coomassie blue. Proteins were electrophoretically transferred from the gel to a nitrocellulose paper using a semidry system according to Towbin et al. (1979). The free binding sites of nitrocellulose were saturated with 5% nonfat dried milk (PBS-milk) for 2 hours at room temperature. The membrane was washed three times in washing buffer for 30 min and the hybridoma supernatants were added (1:1000) for 2 hours at room temperature. The samples were washed three times and then incubated with HRPO conjugated goat anti-mouse IgG (Dakopats, Denmark) for 1 hour at room temperature. The reactions were visualized by diaminobenzidine tetrahydrochloride (DAB, Sigma Chem Co. USA) as substrate.

The sera were boiled for 10 min in SDS sample buffer and were separated by 15% SDS–PAGE using Mini Protean 3 devices (Bio-Rad, Hercules, USA). The gel was transferred to a Hybond ECL membrane (Amersham Biosciences), blocked with 5% non-fat dry milk in PBS for 1 h and incubated with anti-HBX monoclonal antibodies (1:500, 3F6/G10, 4F1/A9) in PBS containing 1% non-fat dry milk. After the membrane was washed three times with PBS, horseradish peroxidase-coupled anti-mouse IgGs (1:5000 Amersham Biosciences) were added and the signal was detected with the ECL Plus Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions, using Kodak X-ray film.

Immunohistochemical characterization

The specificity of monoclonal anti-HBxAg antibody clones was analyzed in different human tissues. Formaldehyde fixed and paraffin embedded tissue sections from clinically verified B hepatitis positive liver biopsies were used as positive controls. HBV negative, normal and pathologic tissues originating from the tissue bank of Histopathology Ltd. were analyzed during the further immunohistochemical characterization of monoclonal anti-HBxAg clones. An IgG2a (anti-CD45RO, clone UCHL-1; Immunotech Inc., France) monoclonal antibody was used as irrelevant, negative control during the whole immunohistochemical investigations.

Retrospective immunohistochemical study

A retrospective study on needle biopsy specimens from patients with acute viral hepatitis (10), chronic hepatitis (45), liver cirrhosis (9) and surgical biopsy specimens from patients with PHC (8) was performed after the selection of the best anti-HBxAg clone (No. 3F6/G10 with IgG2a isotype). The tissue samples were obtained from the Pathology Service of the County Hospital of Baranya in Pécs, County Hospital of Zala in Zalaegerszeg and from the Histopathology Ltd., Pécs. Formaldehyde-fixed, paraffin embedded sections were stained by polyclonal (goat) biotinilated anti-HBsAg (Dacopatts, Denmark) and biotinilated (rabbit) anti-HBcAg (Dakopatts, Denmark) antibodies. The findings were compared to the results of immunohistochemical investigations by monoclonal anti-HBxAg (IgG2a) antibody. Biotin labeled anti-mouse Ig (Dakopatts, Denmark) was used as secondary antibody in the case of anti-HBxAg. The reactions were developed by the HRPO streptavidin–biotin complex method. Immunostaining was visualized by H_2O_2-amino ethyl carbasol (Sigma Chem. Co., USA) substrate and the sections were then counterstained with hematoxylin. Irrelevant mono-

clonal antibodies as the anti-FITC $IgG1^2$ and the anti-UCHL-1 IgG2a (Immunotech Inc., France) were used as negative controls.

Epitope determination of the anti-HBxAg 3F6/G10

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 1ml 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).

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 _g/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, 1010 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/0.1M glycine, pH 2.2. Following neutralization with 2M Tris–base, 10 ml XL1-Blue (O.D.600: 0.5) was infected and plated on LB agar plates containing 50 _g/ml ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with 1011 plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37 ◦C phage were precipitated with 16.7% PEG8000/3.3M NaCl twice and resuspended in TBS. Following the second round of panning 10 ml XL1-Blue (O.D.600: 0.5) was infected with 104 ATU phage, superinfected with 1011 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 _g/ml in blocking solution) for 2 h. Positive clones were visualized with an AP conjugated anti-mouse IgG secondary antibody.

Serum samples

All sera were obtained from the laboratory services of the First Department of Medicine, Medical Center of the University of Pécs and the Division of Virology, Bela Johan National Center for Epidemiology, Budapest, as residual samples after completion of all laboratory testing. The results obtained with sera of patients suffering from HBV positive acute (14) and chronic (80) hepatitis and those taken from healthy HBV carriers (12) were compared with healthy, HBV negative, serum samples (22) and sera from symptomless HBsAg-carrier pregnant women (80). The sera were pretested for HBV by specific ELISA for HBsAg and HBcAg (Dialab, Hungary).

Low pH pretreatment was used in serum samples before the immunoassays to decompose the immunocomplexes and the non-specific aggregates present in sera stored in the frozen state at –20 °C. GST-HBxAg standard and serum samples were diluted in glycine (pH 2.0, 10 min, 37 °C) and than restored to pH 8.0 in Tris-base to a final dilution of 1:10.

Construction of sandwich type ELISA

Assay conditions were optimized by checkerboard titration of recombinant HBxAg-GST standard. Microtitre plates (Nunc, France) were coated with 100 µl of mouse monoclonal anti-HBx (3F6/G10) antibody at a concentration of 10 µg/ml in 0.05 M bicarbonate buffer (pH 9.6) at 4 °C overnight, followed by 60 min incubation at 37 °C. After repeated washing, the plate with 0.15 M phosphate-buffered saline (PBS) non-specific binding sites was blocked by adding 1% gelatin–PBS (300 µl) at 37 °C for 1 h. Then, the plate was washed four times with PBS–Tween 20 (PBST). Following the last wash, GST-HBxAg standard and serum samples were diluted in glycine buffer (pH 2.0, 10 min, 37 °C) and then restored to pH 8.0 by adding Tris-base to a final dilution of 1:10. The plate was incubated at 37 °C for 4 h. After washing, 100 µl of biotin-conjugated mouse monoclonal anti-HBx (4F1/A9) antibody (2 µg/ml) were added to each well and incubated at 37 °C for 1 h and washed again. Then, 100 µl of streptavidin-horseradish peroxidase (Sigma, USA) diluted to 1:3.000 in PBST were added to each well and incubated at 37 °C for 1 h. Finally, the plate was washed, the color reaction was developed by *ortho*-phenylenediamine (oPD) (Sigma, USA), and was stopped by 0.46 M sulfuric acid (100 µl/well). Optical densities (ODs) were read at 490 nm in a plate reader (Thermo Labsystems IEMS Reader MF, Finland). The levels of HBxAg in the serum samples were interpolated from a GST-HBx standard calibration curve using an automatic calculation program (Labsystems Ascent Software 2.4 for IEMS Reader MF).

ELISA on HBx antigen fragments

Microtitre plates (Nunc, USA) were coated with an even amount of antigens relative to the peptide component of each construct (recombinant protein or synthetic peptide fragment) in 3µg/ml concentration dissolved in 0.1M bicarbonate buffer, pH 9.6 overnight at 4°C. Followed by incubation for 1 hour at 37°C. We washed the plates three times with PBS containing 0.05% Tween 20 (Sigma, USA) and PBS-gelatin (0.5%) (Sigma, USA) treatment was used for 30 min to eliminate the unbound antigen and to saturate the free bindings sites of the assay plates. After a washing step serum samples (1:100 diluted in PBS-Tween 20) were added for 1 hour at 37°C. Following the next wash with PBS-Tween 20, HRPO labeled rabbit anti-human IgG and IgM (DAKO, Denmark) was added to the wells in 1:2000 dilution (Diluted in PBS-Tween 20) and incubated for 1 hour at 37°C .The plates were repeatedly washed with PBS-Tween 20. Enzyme acivity was visualised using o-phenylenediamine (Fluka, Germany) and 0.02% H_2O_2 added in 0.1M citric acid buffer (pH 5.0). The reaction was blocked by sulfuric acid (4M) and the results were counted by a microphotometer (Labsystems Ascent Software 2.4 for IEMS Reader MF) at 490 nm wavelength. The measurements were performed in parallel under standard conditions, the samples were applied in triplicate. The quantitative measurements of serum HBxAg concentrations yielded constant values (the inter-assay variability scatters were less than 5%), the HBx specific antibody measurements showed standard tendencies during the repeated measurements.

Cytometric bead array immunoassay

Microparticles were prepared and coupled to monoclonal antibody as previously described (Carson and Vignali, 1999). Briefly, polystyrene beads, 7.5 nm diameter (BD Biosciences USA), were coupled via a covalent linkage based on thiol-maleimide chemistry with monoclonal anti-HBx (3F6/G10) capture antibody according to the labeling kit and the manufac-

turer's protocol. The labeled microbeads were stored for sequential investigations at +4 °C in storage buffer. Before the measurement, 1000 beads per sample were reconstituted in washing buffer by ultrasound (37 kHz, 100 W, 2 min) at room temperature. Each serum sample was pretreated as described in the ELISA protocol. The samples were incubated with 1000–1000 beads for 4 h at 37 °C. Then, the samples were washed and incubated with biotin conjugated anti-HBx (4F1/A9) detecting antibody (2 μg/ml) for 1 h. After three washing steps, the samples were incubated with streptavidin-FITC (DAKO, Denmark) in 1:100 dilution for 1 h at 37 °C. The samples (after three washing steps) were acquired within 1 h using a FACSCalibur flow cytometer (BD Biosciences USA) and list mode files were further analyzed with FCAP Array software (SoftFlow, Hungary). Comparing the mean intensity values of the FITC reporter fluorescence of the samples to a standard curve, the software generates quantitative data on the HBxAg concentration in patients' sera.

Statistical analysis

Statistical analysis was performed by Student's *t* test. The data were analyzed with SigmaPlot software 2000 (SPSS Inc., USA)

In silico homology analysis of HBxAg epitopes

In silico analyses was performed to check for known homologies of 13–26 and 89–94 subsequences of HBxAg (gi:4704317). Subsequences were searched against the non-redundant protein database (database size as of September 2004 = 688,443,072) using the BLASTP2.2.9 program (Altschul et al., 1997) (expect = 20000; Matrix PAM30; gap cost existence = 9; extension = 1; word size = 2). Results were limited by the Entrez option: hepatitis B virus [ORGANISM] AND X protein [PRODUCT] (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Results

1. Mapping of HBxAg immunogenity on computer models and developement of recombinant HBxAg fragments based on predicted data.

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. 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.

2. Anti-HBxAg monoclonal antibodies developed against recombinant and synthetic antigens. Immunoserologically and immunohystochemically characterisation of antibodies.

An anti-HBxAg monoclonal antibody family was developed following the usual way against recombinant hepatitis B virus X protein. Monoclons were selected and characterized immunoserologically and immunohistochemically. Monoclonal IgG2a antibody secreted by

hybridoma clone 3F6/G10 was a like optimal for ELISA, immunoblot and immunocytochemistry. The anti-HBxAg monoclonal antibody recognized (in 5 ng/sample dilution) the recombinant antigen (in 250 ng/sample) as proved by high optical density (OD490 ≥1.000) measured in a simple binding ELISA. 2.3 ng/well antigen concentration (calculated for the X protein) was the minimum antigen level in the optimal measurable range (OD490 ≤0.400). No reactions were found with the fusion partners (GST, and Protein A) or other non-specific mammalian and non-mammalian proteins. Further characterization of anti-HBxAg antibody clone by immunoblot technique showed obvious positivity in the relevant band. The immunohistochemical characterization did not show any positive reaction on different normal human tissues (embryonic and adult) and non-mammalian samples. However, intensive immune reactivity was found in liver biopsy samples from chronic hepatitis B virus carrier patients in different histological and cellular localization. Granular cytoplasmic and nuclear stainings were present in equal density in some cases and an exclusively intense nuclear positivity in some others.

3. Results of immuno-hystochemistry by anti-HBxAg monoclonal antibody on liver-biopsy samples from patients infected by hepatitis B.

Retrospective histopathological analysis was performed on 72 liver biopsy samples of clinically verified hepatitis B virus holding patients. We analyzed the microscopic appearance and the type of intracellular localization of HBxAg immunostaining according to the classification of commonly accepted literature data. The highest number of HBxAg positive cases was found in the group of patients with chronic hepatitis (86.6%) in heterogeneous microscopic appearances. Between 40 to 50% of the biopsy samples showed HBxAg positivity in the groups of acute hepatitis, liver cirrhosis and primary hepatocellular carcinoma. The histologic and cytologic distribution of HBxAg showed various forms in each group. The frequency of positive cells was the highest in chronic hepatitis and PHC, however, the intensity of the immune reactions was ambiguously strongest in the group of PHC samples. Intracellular localization after anti-HBxAg immunostaining showed cytoplasmic and/or nuclear distributions in general, but dominant nuclear positivity was found in PHC. The histological occurrence of labeling with anti-HBxAg monoclonal antibody showed a focal pattern (localized in well-defined groups of HBxAg positive cells between broad negative regions, marked as “L”) in the majority of acute and chronic B hepatitis biopsy samples. Moderate cell membrane staining was found in one chronic B hepatitis case with granular cytoplasmic and heterogeneous nuclear labeling. The cytoplasmic occurrence was granular in some cases, fine granular (diffuse) in the other samples independently from the clinical and histopathologic stages of chronic hepatitis or cirrhosis.. A predominantly nuclear appearance was typical with diffuse histologic pattern in a few chronic hepatitis samples. We found characteristically intense and uniform nuclear immunoreactions with HBxAg antibody in each cell of the entire liver tissue (both in the tumor and in the residual liver) on PHC cases with moderate cytoplasmic labeling. The results of the anti-HBxAg retrospective study on 72 biopsy samples with detailed data of the histologic and cytologic microscopical appearance are summarized. To exclude non-specific nuclear reactions, which could occur in some formaldehyde fixed samples using mouse antibodies, we tested all tissue samples with an irrelevant mouse monoclonal antibody as negative control (anti- UCHL-1 IgG2a) but no false positive immunoreactivity was found. We compared the microscopic immunostaining pattern of monoclonal anti-HBxAg antibody with two commercially available polyclonal antisera against other hepatitis B virus antigens. In the laboratory daily routine the determination of both “s” (surface) and “c” (core) antigens are used for primary diagnosis and as prognostic factors. Our monoclonal antibody showed significantly more sensitive reactions in the group

of chronic hepatitis patients than the HBsAg or HBcAg antibodies. The limited number of the investigated cases in other groups did not allow correct calculations in liver cirrhosis and PHC, but the tendency is clear. The microscopic patterns of HBxAg positivity have been summarized in different histopathological stages of HBV infection-related diseases.

4. Results originated from the epitope mapping of anti-HBxAg monoclonal antibody.

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. This sequence can be found in the primary structure of HBX (amino acids 89–94). We found strong reactions with anti-HBX Mab in GST fusion peptides spanning amino acids 79–143 of the HBX. The anti-HBX Mab recognized only the segment representing amino acids 79–117. No reaction was found when peptides containing sequence 97–136 and 117–143 were tested. According to the alignment of the segments only the sequence 79–94 can possibly contain the epitope bound by the anti-HBX Mab. This calculation further supports the results of the random peptide library screen, which identifies the epitope sequence as LPxxLH (amino acids 89–94 of the HBX).

5. Experimental results of immuno-assays developed to detect the HBxAg and anti-HBxAg antibodies.

The selectivity of HBxAg ELISA was tested on 128 human serum samples: the HBV negative samples (22 healthy control sera) were consistently negativein the assay (the OD490 was in all cases under 2SD of the OD value of the blank and the control sera). The presence of the HBxAg on 106 HBV infected patients was detected by ELISA in 64% of acute patients, in 76% of chronic hepatitis patients and in 50% of the healthy carriers. The cut off range was N4 ng/ml according to the results of the healthy controls. (The previous literature data are suggest a similar range. The concentrations were between 18.2 and 1803.86 ng/ml.

We used the recombinant 10-143 HBxAg and other six recombinant antigen fragments and a synthetic N-terminal peptide fragment (13-26) for mapping the antibodies present in the sera of HBV infected patients. Serum samples of acute (14 patients) or chronic hepatitis (80 patients), symptomless carriers (12 patients) and 22 healthy donors were analyzed for anti-HBxAg antibodies of IgG and IgM isotypes.

The HBxAg serum levels were measured by sandwich type ELISA developed previously (Pal et al., under publication). The measured concentrations ranged in a wide spectrum of 18 – 1800 ng/ml. The averages of serum HBxAg concentrations were higher in acute and chronic hepatitis samples than in symptomless carriers; however, individual variations were also elevated in chronic hepatitis patients.

Demonstrates the distribution of serum titers of circulating anti-HBx antibodies of IgG and IgM isotypes. Individual differences were relatively homogenous except for anti-HBx IgG in chronic hepatitis patients. For both the IgG and IgM isotypes, the strongest anti-HBx response was observed against the longest fragment of the recombinant antigens (10-143). The recombinant C terminal fragment (C1: 79-117) was typically positive in chronic hepatitis patients for both IgG and IgM isotypes. IgG antibodies showed strong binding to the same sequence in acute hepatitis and in symptomless carriers. IgM reaction against the rest of analyzed sequences was slightly positive, with minimal individual variations. Healthy controls proved to be consistently non-reactive to all the antigens included in the study.

Correlations between the HBxAg serum concentration and antibody titers against HBxAg fragments were subjected to regression analysis to unveil hidden correlations among different

parameters and groups. The results of regression analysis performed on HBxAg serum concentrations and the antibodies responding to different HBx fragments (both IgG and IgM) as measured in the sera. Reaction of antibodies belonging to the IgM isotype with all HBxAg fragments showed strong correlations with each other in patients with acute hepatitis. IgG antibodies consistently exhibited highly specific recognition of C1 fragments and accordingly, ignorance of the N terminal end. Also there has been a strong correlation between the serum HBxAg level and the antibodies specific to the longest fragment (X: 10-143).
Among the antibodies detected in chronic hepatitis B patients, we have found a high correlation between IgG type antibody responses in the case of C-terminal fragments, but it was missing in the case of N-terminal fragments. There was a moderate correlation between the serum level of HBxAg and the IgG response against the C-terminal fragments and the longest fragment (X: 10-143). The regression analysis revealed that concerning IgM reaction N- and C-terminal fragments were dominant, but no correlation was found with the antigen concentrations in the sera.

The regression analysis results of symptomless HBV carriers showed close similarities to acute hepatitis B patients. In the case of IgM type immune response, there was a strong correlation of antibody titers against C3 and N-terminal fragments. However, correlations for IgM were less homogenous as compared to patients with acute hepatitis, and correlations were found between the serum HBxAg concentration and C1- and N-terminal fragment-specific IgG production. This latter probably indicates that the forming immune complexes are made up of IgG antibodies and HBxAg.

6 Quantitative analysis of circulating HBxAg *via* sandwich-type flowcytometric micro-bead assay.

The microbead assay was tested with the sera of chronic hepatitis patients (32), sera of symptomless HBsAg-carrier pregnant women (80) and healthy individuals (22). demonstrates characteristic positive and negative samples. The results were compared to the results of the ELISA measurements performed in parallel. Comparative analysis of the two assays showed the same sensitivity and selectivity; the correlation coefficient of the data (measured on the same serum samples) was 98.67%. The microbead assay resulted in similar sensitivity as the ELISA.

Discussion

We have studied the hepatitis B virus X antigen (HBxAg), a protein of high hidrophobicity. The molecular micro-environment that influences immune-recognition including the study of basic physico-chemical characteristics of antigens is one of the most important topics of basic immunology studies. This has a great impact on both biotechnology and routine. The 3D localization of the electrostatic and hydrophobic/hydrophylic regions on the surface of the antigen, determines immune reactivity and plays a crucial role in immune recognition and effector mechanisms. It is generally known that antigens, showing strong hydrophobic features, form non-specific molecular connections and so they are often masked. They are hard to reach for the recognition molecules and cells of immune system and the immunogenicity of hidden epitopes changes counts a lot. We have few data on the antigenicity of masked residues.

Various methods like NMR and X-ray crystallography are applied for mapping epitopes. They are successful in defining epitopes by limited proteolysis followed by mass-spec analysis and ELISA on overlapping peptides synthesized on polypropylene needles. Other methods used random libraries of peptides, recombinant antigen fragments or fragments of

antigens, expressed on the surface of phages; also there are model predictions. In the case of non-linear epitopes or antigens exhibiting unusual physico-chemistry, most of the methods used for epitope-mapping can be applied following corrections. The efficiency of these techniques is increased by probability analysis or through computer modeling of the immunogenic regions of the antigen molecules. For antigens with unusual physico-chemical characteristics the efficiency of regular immune-serological or spectrophotometric applications and the success of computer structure analyses is severely impaired. We have also experienced this phenomenon when studying the epitope specificity of our anti-HBxAg monoclonal antibody:

We had to perfrom epitope mapping since the positive results we obtained during the selection of anti-HBxAg hybridomes using synthetic peptides as a control, were not in the region predicted previously *in silico* (sequences 22-31 and 100-114). According to literature, the entire HBxAg sequence is able to induce the production of antibodies in HBV infected patients and the sequence of 100-114 is probably localized in an immunodominant region.

The epitope mapping using mass-spec analysis did not lead to success in the case of anti-HBxAg (clone No.: 3F6/G10) monoclonal antibody. Later on, we successfully used a random peptide library, consisting of nine amino acid residues, expressed on the surface filamentous phages to determine the sequence of the epitope. The defined epitope sequence ($_{89}$LPKVLH$_{94}$) contains a digestion site for tripsin. Following tripsin digestion, during the first step of the mass-spec analysis, the amount of the epitope sequence decreased. It probably coagulated during the treatments as the protein region, containing the sequence, is hydrophobic. This was the reason of the unsuccessful epitope mapping *via* mass-spec analysis.

Sequences containing the epitopes of our antibodies – clone: 3F6/G10: (89-94 residues) and clone: 4F1/A9: (13-26) – have strong antigenic characters based on the index of Jameson and Wolf (Jameson, 1988). According to Chou-Fasman and Garnier algorithms, that predict the localization of α-helices and β-sheets, the epitopes of our antibodies localize on the border between α-helices and β-sheets. The α-helices and β-sheets are hydrophobic and usually situated inside the proteins. Both sequences belong to the regions of higher flexibility of the HBxAg molecule based on the Karplus and Shultz index of flexibility. According to this algorithm these sequences are located on the surface that suggests their involvement in the immune response.

The technique of producing HBxAg and a-HBxAg monoclonal antibodies able to specifically recognize the antigen is difficult because of the unique physico-chemical characters of the protein since 54% of the protein is made up from hydrophobic residues. The synthetic production of the whole-protein is hard as well, because HBxAg precipitates in aqueous solution; also the isolation of HBxAg from HBV infected human samples needs special procedures. Earlier the MS2-HBxAg and most often the GST-HBxAg constructs were used for the production of recombinant HBxAg. We studied the usability of GST as a carrier molecule compared to numerous antigen constructs. It has been proven during our extensive study, that GST-HBxAg has suitable parameters to use it as a reagent to test antibodies and develop assays.

We were successful in developing a family of monoclonal antibodies using recombinant HBxAg and the parallel characterization (immuno-serological and immunohistochemistry) of clones. This family can be used on routine histological samples embedded in formol-paraffine for immunohistochemistry studies.

According to numerous data in the literature, if the HBxAg is expressed in patients carrying the HBV genome, it has clinically significant prognostic value. The HBxAg immunohistochemistry studies published in the literature were mostly carried out using polyclonal and in some cases monoclonal antibodies. Data clearly shows that HBxAg serves

as a common marker in the cases of viral hepatitis B liver infections (acute, chronic, cirrhosis hepatitis) and PHC cases.

We studied the presence of HBxAg on more than one hundred paraffinated histological slides. The patients infected by hepatitis B virus were categorized in the following types based on the pathologic category of liver biopsy: acute hepatitis, chronic hepatitis, cirrhosis and primary hepatocellular carcinoma. We found the localized labeling of X-antigen in defined groups of cells with dominant cytoplasmic and weak nuclear labeling in acute B hepatitis during our retrospective immunohistochemistry studies. We found highly extended, granulated, cytoplasmic and nuclear positive staining besides the localized presence of strong positive regions in chronic hepatitis B samples. We found positive cell membranes in a chronic hepatitis B case. We were also able to detect decreased cytoplasmic presence of X antigen besides the clear nuclear positive signals in cirrhosis cases. We found strong nuclear labeling comprising the entire liver in PHC cases.

Different research groups found different frequency of HBxAg positive signals depending on the population studied and anti-HBxAg antibodies used. In the cases of cirrhosis patients the nuclear labeling was 70%, in contrast to the 5-15% frequency of nuclear staining in chronic hepatitis cases. This difference in the localization may be indicative for the binding of HBxAg to different cell compartments depending on the type of chronic liver disease hereby the control mechanism of transactivation may be different as well. HBxAg in the cytoplasm presumably influences signal transduction pathways, whereas the nuclear localization may refer to the role of transcriptional transactivation of HBxAg. It may be hypothesized that in the early phase of infection the virus activates different mitogen signals in the cytoplasm and later on, it makes direct contact with different transcription factors in the nucleus.

Our results from immunohistochemistry correlated well with the data from literature both in chronic hepatitis and PHC groups.

By using the monoclonal antibodies developed in our lab we could clarify that the HBxAg could be present in large numbers in infected liver cells not only in the cytoplasm but also in the nucleus. We also made a novel observation: by using our monoclonal antibody the HBxAg immunoreactivity changed depending on the intracellular localization. During the chronic hepatitis B infection the increase of the positive HBxAg signals in the nucleus may indicate the deterioration of the prognosis of the disease and the development of primary hepatocellular carcinoma.

Our monoclonal antibody (clone: 3F6/G10 epitope: 89-94 HBxAg sequence) binds to the same sequence of HBxAg as the XAP1/DDB1 (a protein involved in DNA repair). HBxAg-DDB1 binding inhibits the function of DDB1 protein, cell proliferation, influences the viability of cells and increases HBV replication. The HBxAg 89.-125. interval is an important immunodominant and functional region of human pathogen HBV. According to the prediction, our antibody binds to the 89-94 surface determinant of the antigen. We can declare that our antibody binds to specific HBxAg sequences with high selectivity and the epitope recognized by our antibody has functional importance.

We consider our immunohistochemistry studies significant because we were succesful in producing a monoclonal antibody with an epitope specificity that gives applicable information of the mechanism and possible prognosis of HBV infection even at light-microscopy level.

Although techniques of molecular biology can be applied to detect HBxAg but conventional immunoligical assays are needed to manage high numbers of measurements in routine laboratory services.

We managed to develop two monoclonal anti-HBxAg antibodies with high affinity. These monoclonal antibodies, clone 3F6/G10 isotype IgG2a (epitope: 89-94 residues – gripper antibody in our assay) and clone 4F1/A9 isotype IgG1 (epitope:13-26 residues, detection

antibody in our assay) were described earlier. The following data from the literature are known to characterize the epitope of the latter antibody:

During the investigation of the pathomechanism of HBV imoprtant N terminal regions were also described (1-20 and 13-26). The possible function of XAP2 bound to this region is the inhibition of the transactivator effect of X antigen.

We studied 208 hepatitis B positive human sera with our anti-HBxAg sandwich ELISA method during the comparative serologic investigation. Besides the determination of the amount of HBxAg antigen present in the sera we studied the ratio of natural antibodies produced against the different epitopes of X protein with the help of HBxAg fragments. We were the first to use quantitative methods to study the relationship between disease-stage the amount of circulating antigen and the pattern of epitopes. We applied a long-lasting and widely used correlation analysis with the help multiparametric data management for the evaluation of large number of immunoserologic samples. We succeded in defining well characterized prognostic groups with the help of our matrix statistics method. The amount of circulating antigens in patients in the stage of acute phase of hepatitis B infection shows a homogenic picture/ distribution within the group, with its value between 500-1000 ng/ml. The IgM type immune response has no specific region at this stage of infection but during the IgG type response the C1 (79-117) region becomes significant besides the observed N-terminal recessivity.

We experienced a great spreading and characteristic difference in the amounts of HBxAg within the chronic group, which presumably refers to the persistent and active character of the infection. Investigating the immune response in the chronic stage our results shows IgG dominance besides C- terminal (C-1 region) preference. In addition we can observe an increase of N-terminal IgG type immune recognition. In those patients, where the negative regulatory effect of the N-terminus appears the chronic infection may turn into persistent. In those cases where the immune response against the N-terminus is negligible or doesn't exist at all, it is highly likely that the chronic infection becomes active. In the IgM type immune response, compared to the acute stage, the homogenicity disappears, and the C-terminal dominance becomes significant. Based on our results we can declare that there is a significant link between the expression level of HBxAg and the intensity of immune response against the specific C1 (79.-117. residues) region in the chronic stage of hepatitis B infection. The C-terminal region of HBxAg has a transactivator fuction. The strong immune response against this region and the strong expression of X antigen may indicate the malignant improvement of hepatitis B infection.

We found characteristic IgM type immune response with significant C-terminal and moderate N-terminal correlations in patients carrying the disease without symptoms. The heterogeneity of IgM response can be observed compared to the acute group. Moderate connection can be shown between the IgG type immune recognition against C1 and N-terminal and the amount of HBxAg in the serum. (This latter probably indicates that the immune-complexes consist of IgG antibodies and HBxAg.) This correlation indicates the inhibition of transactivation modifying effect of HBxAg and can play a role in the development and conservation of an asymptomic stage.

The generally used immunoserological experimental techniques are feasible for individual measurements. The abilty to define several parallel parameters from the same samples may be relevant in the clinical routine diagnostics and research. One possible choice is the recently developed flow-cytometry based, micro-bead technology. Polystyrene micro-beads are used as carriers in several immune-assays. The measurements applying flow-cytometry are optimal for the quick preliminary analysis of numerous samples.

Through the development of our sandwich-type anti-HBxAg ELISA and its adaptation we offer an immunoserological micro-bead assay for flow-cytometry capable of micro-analysis.

We also performed the comparative study of these two systems on human serum samples of the same origin. The results show strong correlation.

From a technical point of view, this study is an adaptation that can be used to reveal new correlations. The developed bio-statistical panel can help us in clinical and epidemiological analyses.

Our immunoserological measurements on 208 clinically verified serum-samples support the possible role of the hepatitis B virus protein X in the pathomechanism of HBV and suggest the use of HBxAg as a prognostic marker.

Summary

Hepatitis B virus (HBV) is a frequent human pathogen that belongs to the hepadnaviridae family. According to the study of the World Health Organization (WHO), 400 million chronic hepatitis B infected patients lived on the globe at the millennium. The number of dead due to complications of HBV infection fluctuated between 500 000 and one million in the last decade. The main importance of HBV infection is that in the case of chronic carriers, the chance of the developing primary hepatocellular carcinoma (PHC) increases 200 folds. It is accepted that the X antigen of hepatitis B virus (HBxAg) coded by the fourth "open reading frame" of the virus, plays a crucial role in the development of PHC. This antigen is extremely conservative, the 17kDa protein is very hydrophobic. 80 (54%) of the 154 residues are hydrophobic that represents a physico-chemical structure unique in biological systems. The HBxAg is a multifunctional regulatory protein that modulates the function of many transcription factors. According to data in the literature the expressed HBxAg has fundamental prognostic value in the clinical diagnosis in patients carrying the HBV genome.

The complication of indicating HBxAg originates from its strong hydrophobic character. It is well known that it makes non-specific molecular interactions, it is often masked and rarely accessible for detecting antibodies. Specific treatment of the studied samples is needed for its detection.

We had two major goals during this work. In the first step we produced monoclonal antibodies that interact with the X antigen of hepatitis B virus. This was necessary to make further studies of the virus protein, such as immunological-mapping, immune-serology and immune histochemistry. The other goal was to develop an immuno-serological diagnostic for routine clinical laboratories. No such antibodies and serological assays are currently commercially available.

We were successful in developing a family of monoclonal antibodies by using recombinant HBxAg that was ready to use on routine histological samples embedded in formol-paraffine for immunohistochemistry studies. Their combined use enabled us the quantitative analysis of human sera samples.

Our antibodies bind to functional immuno-determinants. One of our antibodies (clone: 4F1/A9) binds to the N-terminal region (13-26 residues) of HBxAg like the XAP2, that has the probable function of inhibiting the transactivation effect of X antigen. The epitope sequence of our other antibody (clone: 3F6/G10) is located in the DDB1 and HBxAg binding (docking) sequence (DDB1 is a protein involved in DNA repair). The HBxAg-DDB1 connection inhibits the function of DDB1 and through this, modulates the viability and proliferation of cells and increases the HBV replication.

We found specific distribution of positive intracellular HBxAg labeling during our immuno-histochemistry studies. Dramatic differences have been demonstrated in the intensity of positive staining between the cytoplasm and nucleus during the different stages of hepatitis

B infection. It could be concluded in general that while nucleic positive signals were dominated during liver cirrhoses and PHC, in acute hepatitis mainly the cytoplasm showed (granular) positive signals. During chronic hepatitis, intensive (diffuse) staining was manifested both in the nucleus and the cytoplasm. During chronic hepatitis B infection the increase in the percentage of HBxAg-positive nuclei may indicate the relapse of the disease and the development of primary hepatocellular carcinoma.

We performed serological studies by different methods: combined application of our monoclonal antibodies, ELISA, using the fragments of recombinant HBxAg, developed by our *in silico* predicted data, microbead technique feasible for multi-parametric studies.

While the synthetic peptide sequences (chosen by our predictive studies) that have high antigen values were recognized by our polyclonal anti-HBxAg antibodies, the anti-HBxAg antibodies originated from our hybridoma clones did not. We used different methods to perform epitope-mapping. We were successful in determining the amino-acid sequence recognized by our antibodies with the help of peptide library expressed on filamentous phages. The defined regions of the HBxAg, recognized by our pre-chosen antibodies, interact with the regions playing important role in the course of the disease therefore they are appropriate for prognostic studies.

We were able to perform quantitative study on a large number of samples with the help of developed ELISA systems for the first time. In this experiment we reveal the connection between the amount of circulating HBxAg and the titer of different antibodies produced against the different regions of the antigen. To explore the correlations we processed 80 samples of sera (sixteen data points per sample) during our studies. We found new data to define the prognosis of the disease based on the bio-statistic process: there is a significant correlation between the high titer of IgG antibodies against the C1 epitope (amino acid sequence 97-117 HBxAg) and the high amount of circulating HBxAg in the chronic stage of hepatitis B virus infection. According to literature data the C terminus of HBxAg has a transactivator function. The strong immune response against this region and the elevated expression of the X antigen may indicate the malignant development of the hepatitis B virus infection.

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