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ARTICLE

Comprehensive Regression Analysis of Hepatitis B Virus X Antigen Level and Anti-HBx Antibody Titer in the Sera of Patients with HBV Infection

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Although the pathogenetic significance of hepatitis B virus x protein (HBxAg) in chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma has already been studied, the comparative analyses of both the actual serum HBxAg levels and antibody production against various HBx epitopes have been examined to lesser extent. We have simultaneously investigated the relationship between antibody production (IgG and IgM) against the HBxAg fragments and HBxAg level in the sera of patients with acute (14) or chronic hepatitis (80) and symptomless carriers (12). A recently developed sandwich-type ELISA was used for the quantitative measurements of HBxAg. Overlapping recombinant and synthetic antigens were used to map the fine epitope specificities of circulating anti-HBx antibodies. In acute hepatitis, we have found high and homogenous correlation in the IgM type immune responses against all the examined HBxAg regions. Moreover, strong correlation has been observed between IgG type immune responses to a characteristic C-terminal region (C1: 79-117) and the longest fragment (X: 10-143). Moderate correlation has been found between HBxAg concentration and the IgG type anti-HBx antibody levels against C-terminus of HBxAg in patients with chronic hepatitis. In the case of symptomless carriers, there were also demonstrable associations in the immune responses against the C-terminal sequences; however, significant correlations were found for antibody production against the N-terminal region as well. The examinations show that the C-terminal sequence, responsible for transactivation, promotes an efficient IgG antibody response in all three groups of patients, whereas the negative regulator N-terminal part of the HBxAg molecule for the most part does not trigger antibody production. This suggests that the immune responses against various – biologically active – epitopes of the HBxAg may have a different role in the pathogenesis of hepatitis and may be used as prognostic markers in human HBV infections. (Pathology Oncology Research Vol 12, No 1, 34–40)

Key words: Hepatitis B virus, HBxAg, immunoserology, epitope mapping, antibody response

Introduction

Hepatitis B virus (HBV) infection causes severe and permanent public health problems worldwide; its incidence is 4-5% in average with strong geographical differences. Chronic hepatitis, liver cirrhosis and primary hepatocellular carcinoma cause more than half a million deaths every year according to various morbidity and mortality surveys.[9,14,16] Hepatitis B perinatal transmission is close to 50% and may result in a lifelong symptomless carrier state with high epidemiological risks.[15]

HBV has one of the shortest viral genomes and its viral cycle is quite unique too. The X gene is the smallest coding unit of the HBV genome, encoding a 17-kD polypeptide (pX) known as the HBx antigen (HBxAg) consisting of 154, mostly hydrophobic amino acids.[3,5,21] Different

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epitopes of the HBx antigen have various biological functions. Several experimental data reflect the basic importance of HBxAg in the development of chronic hepatitis, liver cirrhosis and primary hepatocellular carcinoma. The pathogenetic roles of different epitopes in disease formation and in the antiviral immune response have intensively been studied.[5,6,13,14,18-20] However, due to technical difficulties, to date no comparative analysis has been performed that would correlate serum HBxAg level with the specific antibody levels produced against different epitopes of the antigen.

Our study is based on a sandwich-type ELISA developed previously for the quantitative determination of serum HBxAg level by a monoclonal antibody pair.[16] Also, we have constructed an overlapping epitope library for epitope mapping of the reacting antibodies. The hidden correlations between the isotypes of reacting antibodies specific to various epitopes of HBxAg and the serum concentration of the antigen were revealed by statistical methods. We investigated the sera of patients with acute and chronic hepatitis and symptomless carriers. For the different entities of the disease, specific patterns of both the targeted epitopes and the isotypes of the reacting antibodies were found. A well-characterized C terminal part of HBxAg was found to be the specific target of IgG-type antibody response.

Materials and Methods

Antigens

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

Construction of HBx recombinant plasmids

Recombinant X antigen (amino acids 10-143). The development of the recombinant construct of HBxAg has been previously described.[10] Briefly, the truncated X gene was obtained by cutting 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-terminus, derived from the pHSG 395 and pGEX-3X plasmids, respectively.

N-terminal fragment (amino acids 10-90) of HBxAg. The original plasmid[2] (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, 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 in the HBV genome) and *EcoR*I (linker sequence of the vector), blunt-ending with Klenow fragment of DNA polymerase I, followed by ligation.

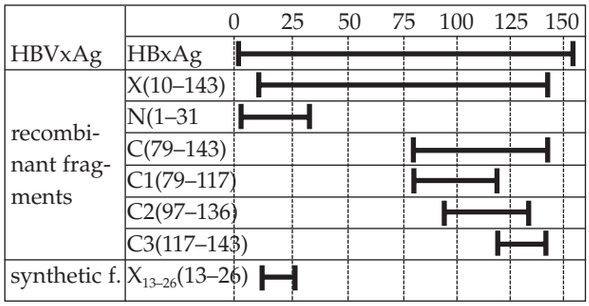

Figure 1. *The 154-aa HBxAg sequence has seven regions. Schematic alignment of the six recombinants (X: 10-143, N: 1-31, C: 79-143, C1: 79-117, C2: 97-136, C3: 117-143) and the synthetic (X_{13-26}: 13-26) fragment. Fragments are represented by dark bands.*

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.[16] 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 DH5a, 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.[10] *Figure 1* summarizes the location of the analyzed HBxAg fragments in the recombinant and synthetic protein.

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.

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, Béla Johan National Center for Epidemiology, Budapest, as residual samples after completion of all laboratory testing. Results obtained with the sera of 14 patients suffering

from HBV-positive acute hepatitis and 80 with chronic hepatitis, as well as with sera taken from 12 healthy HBV carriers were compared with those of 22 healthy – HBV-negative – serum samples. Sera were pre-tested for HBV positivity by HBsAg- and HBcAg-specific ELISA (Dialab, Hungary).

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

Construction of sandwich-type ELISA for the determination of HBxAg concentration

Assay conditions were optimized by checkerboard titration of recombinant HBxAg-GST standard. A microtiter plate (Nunc, USA) was 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. Following repeated washing with 0.15 M phosphate-buffered saline (PBS), non-specific binding sites were blocked by adding of 1% gelatin-PBS (300 µl) at 37°C for one hour. The plate was then washed four times with PBS-Tween 20 (PBST). Following the last wash, GST-HBxAg standard and serum samples were diluted in glycine buffer at pH 2.0 (10 min at 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 four hours. After washing, 100 µl of biotin-conjugated mouse monoclonal anti-HBx (4F1/A9) antibody (2 mg/ml) was added to each well and incubated at 37°C for 1 hour and washed again. Then, 100 µl of streptavidin-horseradish peroxidase (Sigma, USA) diluted to 1:3000 in PBST was added to each well and incubated at 37°C for 1 hour. Finally, the plate was washed and the color reaction was developed by adding the substrate buffer solution containing ortho-phenylenediamine (oPD) (Sigma). The color reaction was stopped by adding 0.46 M sulfuric acid (100 µl/well). Optical densities (ODs) were read at 490 nm in an ELISA plate reader (Thermo Labsystems IEMS Reader MF, Finland). The levels of HBxAg in serum samples were interpolated from the GST-HBx standard calibration curve using an automatic calculation program of the microphotometer (Labsystems Ascent Software 2.4 for IEMS Reader MF).[17]

ELISA on HBx antigen fragments for the determination of anti-HBx antibody concentrations

Microtiter plates (Nunc) were coated with an even amount of antigens relative to the peptide component of each construct (recombinant protein or synthetic peptide fragment) at a concentration of 3 µg/ml dissolved, in 0.1 M 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 PBST, and PBS-gelatin (0.5%) (Sigma) treatment was used for 30 min to eliminate the unbound antigen and to saturate the free binding sites of the assay plates. After a washing step, serum samples, diluted 1:100 in PBST, were added for 1 hour at 37°C. Following the next wash with PBST, HRPO-labeled rabbit anti-human IgG and IgM (DAKO, Denmark) was added to the wells at a dilution of 1:2000 in PBST, and incubated for 1 hour at 37°C. The plates were repeatedly washed with PBST. Enzyme activity was visualized using oPD and 0.02% H_2O_2 added in 0.1 M citric acid buffer, pH 5.0. The reaction was blocked by 4 M sulfuric acid, and the results were counted by a microphotometer (Labsystems Ascent Software 2.4 for IEMS Reader MF) at 490 nm. The measurements were performed 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.

Statistical analysis

Statistical analysis of the collected data was done using an IBM PC, running SPSS for Windows, release 9.0 (SPSS Inc., Chicago, USA). A linear regression analysis of the ELISA results was done for all three groups and all variables. Absolute regression values under 0.4 were rejected, as well as when the P value exceeded 0.05.

In silico homology analysis of HBxAg epitopes

In silico analyses has been performed to check for known homologies of subsequences 13-26 and 89-94 of HBxAg (gi: 4704317). Subsequences were searched against the non-redundant protein database (database size as of September 2004 = 688,443,072) by BLASTP2.2.9 program.[1] (Expect = 20000; Matrix PAM30; Gap cost Existence = 9; Extension = 1; Word size = 2). Results were limited by Entrez option: hepatitis B virus [ORGANISM] AND X protein [PRODUCT] ().

Results

We used the recombinant 10-143 HBxAg, 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 (*Figure 1*). Serum samples of acute (14 patients) or chronic hepatitis (80 patients), symptomless carriers (12 patients) and 22 healthy donors

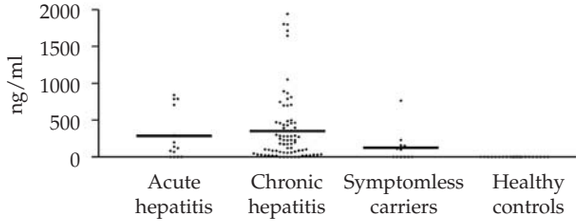

Figure 2. *Quantitative detection of HBxAg in hepatitis B patient sera by sandwich-type ELISA. Sera from HBV-positive acute (n=14) and chronic (n=80) hepatitis patients and those taken from symptomless carriers (n=12) were compared with healthy – HBV-negative – serum samples (n=22). (The "0 ng/ml" level is calculated by the Labsystems Ascent Software 2.4 automatically by the measured OD 490 values.)*

were analyzed for anti-HBxAg antibodies of IgG and IgM isotypes.

The HBxAg serum levels were measured by a previously developed sandwich-type ELISA.[17] 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 (*Figure 2*).

Homology searches with subsequence 13-26 of HBxAg yielded 442 positive blast hits. In 50 cases there was one, in 2 cases there were two amino acid differences compared to the original query sequences. In subsequence 89-94, we retrieved 436 blast hits. In 42 cases there was only one different amino-acid, in 45 cases there were two different amino-acids as opposed to the query sequence. All the hits represent different HBxAg variants. In our interpretation this means that the examined subsequences of HBxAg are highly conserved.

Figure 3 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. *Tables 1-3* show 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 hepati-

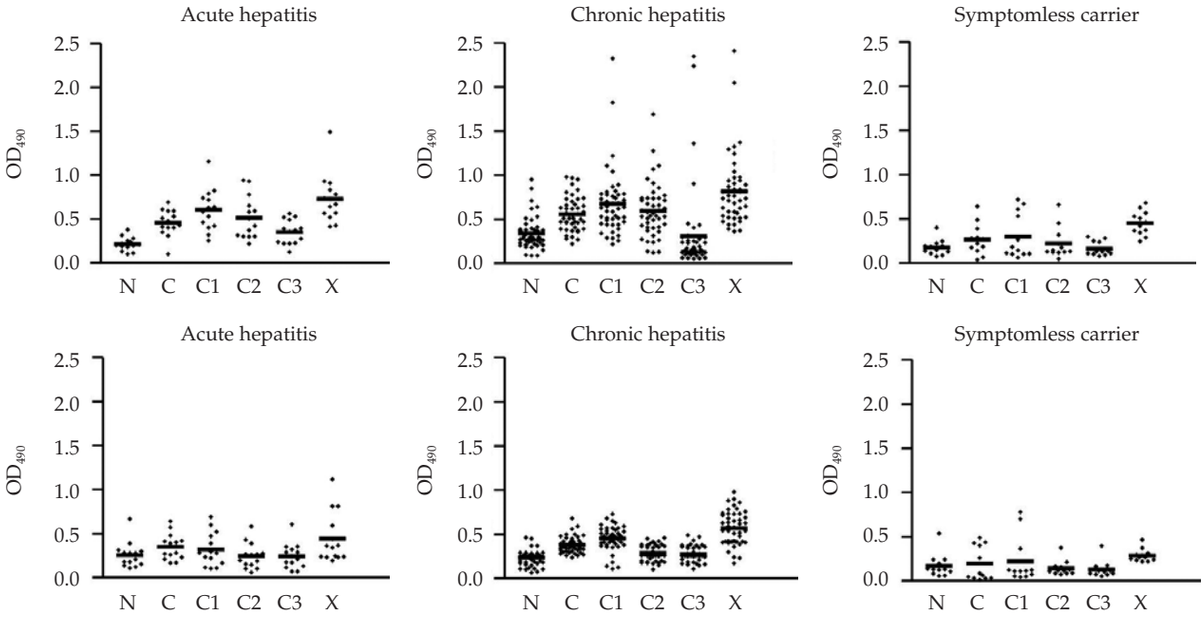

Figure 3. *Results of ELISA using synthetic and recombinant HBxAg fragments for the detection of anti-HBxAg antibodies in the sera of HBV patients (confirmed acute B hepatitis, chronic B hepatitis and symptomless carriers of HBV).*

Table 1. **Regression analysis of ELISA results for HBxAg and anti-HBxAg antibodies in the sera taken from acute hepatitis patients**

				IgG								IgM			
	HBxAg	N	C	C1	C2	C3	X	X^{13-26}	N	C	C1	C2	C3	X	X^{13-26}
HBxAg	1	–	–	–	–	–	0.845	–	–	–	–	–	–	–	–
N		1	–	–	–	–	–	–	–	–	–	–	–	–	–
C			1	–	0.686	–	–	0.539	–	–	–	–	–	–	–
IgG C1				1	–	–	0.976	–	–	–	–	–	–	–	–
C2					1	–	–	–	–	–	–	–	–	–	–
C3						1	–	0.634	–	–	–	–	–	–	–
X							1	–	–	–	–	–	–	–	–
X^{13-26}								1	–	–	–	–	–	–	–
N									1	0.909	0.981	0.996	0.990	0.868	0.907
C										1	0.949	0.922	0.951	0.970	0.956
C1											1	0.986	0.995	0.915	0.945
IgM C2												1	0.995	0.883	0.915
C3													1	0.917	0.940
X														1	0.965
X^{13-26}															1

Only correlation coefficients above 0.4 are presented, P=0.05. High correlation coefficients (above 0.800) are highlighted with grey background. The first line and column show the amount of HBxAg.

tis (*Table 1*). 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 (*Table 2*), 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

Table 2. **Regression analysis of ELISA results for HBxAg and anti-HBxAg antibodies in the sera taken from chronic hepatitis patients**

				IgG								IgM			
	HBxAg	N	C	C1	C2	C3	X	X^{13-26}	N	C	C1	C2	C3	X	X^{13-26}
HBxAg	1	–	0.626	0.648	0.607	0.627	0.664	–	–	–	–	–	–	–	–
N		1	0.413	0.518	–	–	0.540	–	–	–	–	–	–	–	–
C			1	0.843	0.799	0.817	0.753	–	–	–	–	–	–	–	–
C1				1	0.896	0.952	0.846	–	–	–	–	–	–	–	–
IgG C2					1	0.930	0.781	–	–	–	–	–	–	–	–
C3						1	0.839	–	–	–	–	–	–	–	–
X							1	–	–	–	–	–	–	–	–
X^{13-26}								1	–	–	–	–	–	–	–
N									1	0.469	0.943	0.967	0.965	0.414	–
C										1	0.420	0.466	0.467	0.526	–
C1											1	0.938	0.955	–	–
IgM C2												1	0.986	0.473	–
C3													1	0.467	–
X														1	0.477
X^{13-26}															1

Only correlation coefficients above 0.4 are presented, P=0.05. High correlation coefficients (above 0.800) are highlighted with grey background. The first line and column show the amount of HBxAg.

Table 3. **Regression analysis of ELISA results for HBxAg and anti-HBxAg antibodies in the sera taken from symptomless HBV carriers**

					IgG							IgM			
	HBxAg	N	C	C1	C2	C3	X	X^{13-26}	N	C	C1	C2	C3	X	X^{13-26}
HBxAg	1	0.651	–	0.897	–	–	–	–	–	–	–	–	–	–	–
N	1	–	0.732	0.732	–	–	–	–	–	–	–	–	–	–	–
C			1	–	–	–	–	–	–	–	—	–	–	–	–
IgG C1				1	–	–	–	–	–	–	–	–	–	–	–
C2					1	–	–	–	–	–	–	–	–	–	–
C3						1	–	–	–	–	–	–	–	–	–
X							1	–	–	–	–	–	–	–	–
X^{13-26}								1	–	–	–	–	–	–	–
N									1	–	0.775	–	0.825	0.651	–
C										1	–	0.689	0.641	–	0.655
C1											1	0.657	0.802	–	–
IgM C2												1	0.933	0.781	0.766
C3													1	0.696	0.615
X														1	–
X^{13-26}															1

Only correlation coefficients above 0.4 are presented, P=0.05. High correlation coefficients (above 0.800) are highlighted with grey background. The first line and column show the amount of HBxAg.

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 (*Table 3*) 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.

Discussion

The need for a relevant prognostic serum marker and a well-characterized molecular target for biotherapies (e.g. antigen-specific vaccination) in HBV infection have increased recently. Laboratory detection of HBs, HBe and HBc antigens is appropriate for general diagnostic value; however, immunopathological studies support the basic role of the HBx antigen in the pathogenesis of chronic hepatitis B and primary hepatocellular carcinoma.

HBxAg appears in the serum during peak periods of active viral replication as evident from immunoserological analysis of chronic hepatitis B patients.[4,17] The antigenicity of HBxAg protein has been mapped earlier by 15-mer overlapping peptides using the sera of HBV-positive patients. The immunodominant epitopes were located close to the C-terminal end (85-110 sequences) of HBxAg.[20]

Several research groups have identified numerous T-cell epitopes using synthetic polypeptides. Most of them were located to the carboxyterminal part of the HBxAg protein.[6,7] In 1994 Murakami suggested the distinction of functional domain structures of HBxAg. A regulatory domain and a transacting domain have been proposed. The physiological role of a highly conserved nine amino acid sequence (132-140) on the C terminal part was described as the regulator of HBV replication.[19] The HBx 58-140 residues are involved in transactivation.[8] This segment was described as the regulator of AP-1 binding activity in the nucleus and a regulator of the MAPK pathway activation in the cytoplasm.

The N-terminal (1-20) region of HBxAg is the most conserved region among mammalian hepadnavirus genomes; its role has been described as a negative regulator of transactivation.[1]

The pathophysiological roles of different epitopes have been mapped by the use of overlapping synthetic peptide libraries and recombinant antigen fragments in this study; although the complex analysis of the antibody response against these epitopes was not performed. Our ELISA assay developed previously for the large-scale quantitative measurement of circulating HBxAg made the study of fine correlations possible between the serum concentra-

tion of the X antigen and the antibody response against the HBxAg fragments in different stages of HBV infection.

In accordance with our previous observations, we have found homogenous correlations in IgM type immune responses in acute hepatitis B, along with a significant correlation in IgG response against the well-characterized C-terminal epitope (C1: 79-117) and the longest fragment (X: 10-143). The key importance of this sequence is proved by the increased antibody production of both IgG and IgM isotypes in chronic hepatitis B. Regression analysis showed strong correlation between the serum concentration of HBxAg and the IgG antibody production against C1 in chronic hepatitis B and symptomless carriers. Our observation is in harmony with the data of Kumar et al.[8] who described the C-terminal region of the HBxAg as a major target of efficient T-cell responses.

The fine mapping of the epitopes by recombinant antigen fragments localized the relevant sequence to the region between 79-117 amino acids. This is a pathophysiologically active part of the HBxAg that is responsible for transactivation. On the other hand, only insignificant antibody responses could be detected against the negative regulator N-terminal part of the HBxAg in acute and chronic hepatitis patients. The intense IgG production against C1 and the low antibody responses against the N-terminal sequences suggest a causal humoral immune response in acute hepatitis B. In parallel with the dominance of the C1 fragment, other C-terminal epitopes (C2, C3 and the whole C) are also frequent targets of the HBxAg-specific humoral immune response. The significant antibody responses against N terminal sequences in the symptomless carriers may also have pathogenetic consequences by targeting the negative regulatory region of the HBxAg molecule.

The serological examination of the HBxAg and anti-HBx antibody came to spotlight again the past few years. The use of HBxAg as a prognostic marker, as suggested by literature, is the rationale for performing additional systematic investigations.[5,6]

By means of regression analysis of serological results we bring further improvements compared to our previous results. In this study we clearly demonstrated that there is an important connection between the HBxAg level and the anti-HBx antibody titers in the sera of patients infected with HBV.

Our work represents a major contribution to the issue of using HBxAg as a prognostic marker, and provides valuable information related to the pathogenesis of the virus and future vaccination programs.

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