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Full Length Research Paper

Analysis on clinical features and immunity in chronic hepatitis B virus infected patients with low-level HBsAg

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To understand clinical features and immunity of chronic HBV-infected patients with high or low level HBsAg, the differences of serum two- component-determined circulating immune complexes (TCIC) and peripheral blood lymphocyte subsets between these patients were analyzed. 126 patients with chronic hepatitis B virus infection were divided into two groups including 44 with low-level HBsAg and 82 with high-level HBsAg, and three phases including non-active phase, immune active phase and immune tolerant phase by level of HBsAg and natural history of chronic hepatitis B virus infection. Antibody capture-ELISA method and flow cytometry were used to assay serum TCIC and peripheral blood lymphocyte subsets in 44 patients with low-level HBsAg (Group A), 82 patients with high-level HBsAg (Group B) and 22 healthy volunteers (Group C), respectively, and the results were analyzed and compared. Among these 44 patients in Group A, 40 patients (90.9%) were stable in non- active phase and the positive rate of HBsAg/IgG-CIC was the highest, accounting for 15.9% (7/44), while the positive rate of HBsAg/C3-CIC was the highest in Group B, accounting for 46.3% (38/82). The positive rates of serum TCIC, A value and non-active $CD3^+CD8^+$ percentages in Group A were all lower than those in Group B with corresponding phases (P<0.01-0.05), however, the percentages of $CD4^+/CD8^+$ were higher than those in the corresponding phases of Group B(P<0.01) . Patients with low-level HBsAg were correlated to low capability of TCIC synthesis and elimination and there was a low dose-induced immunotolerance phenomenon.

Key words: Immune complex, chronic HBV infection, HBsAg, lymphocyte subsets, immunotolerance.

INTRODUCTION

Population with low-level HBsAg in diagnosis of infectious diseases has been attracting more and more attention of clinical laboratories and epidemiological experts (Raafat et al., 1998; Ijaz et al., 2001; Gall and Nielsen, 2001; Weber, 2005; LI, 2006). Detection of low-level HBsAg has brought new challenges to clinical laboratory testing (Chen and Wu, 2002; Dufour, 2006; Satoh et al., 2008) and new thinking to clinical diagnosis and treatment (Hou et al., 2005; Bhatti et al., 2007).

In epidemiology, it is worth to discuss the differences of distribution, clinical features, serological performance, serotype, genotype and host immune function between the low-level HBsAg infected individuals in the natural population and chronic hepatitis B virus (HBV)-infected patients and high-level HBsAg infected patients (Chinese Journal of Hepatology, 2005; Thompson et al., 2007). HBsAg level and natural history of chronic HBV-infected patients were studied in this study. Antibody capture-ELISA method and flow cytometry (FCM) were used to assay serum two-component-determined circulating immune complexes (TCIC, including HBsAg/IgG-CIC, HBsAg/IgA-CIC, HBsAg/IgM-CIC as well as HBsAg/C3-CIC) and lymphocyte subsets (T lymphocyte subsets, B cells and NK cells), in order to understand the clinical features and main immunities of patients with low-level

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HBsAg.

MATERIALS AND METHODS

Materials

A total of 148 serum specimens of chronic HBV-infected patients were randomly collected from daily out-patients, hospitalized patients and health examination patients. Among these, there were 44 serum specimens of low-level HBsAg (Group A, on the basis of the standard provided by Clinical Test Center of Medical Command, serum HBsAg level <5 ug/L was defined as low level and the content of HBsAg <72 ± 6.8 S/N, as assayed by Abbott Axsym immunology analyzer and confirmed by neutralization test after ELISA screening), excluding the specimens of low-level HBsAg in the early stage of HBV infection or the stage of converting of HBsAg/anti-HBs as found by follow-up, and there were 40 cases in non-active phase or low/non-replicative phase (non-active phase), 2 cases in immuno-active phase and 2 cases in immunotolerance phase according to the natural history (Chinese Journal of Hepatology, 2005; Thompson et al., 2007). There were 82 cases of serum specimens of high-level HBsAg (Group B, HBsAg content >79S/N, including 35 cases in non-active phase, 37 cases in immuno- active phase and 10 cases in immunotolerance phase). Besides, 22 specimens with totally negative serologic HBV markers (HBV M) were collected from health examination patients as control (Group C). All the above subjects were not associated with infection history of HIV or hepatitis virus of other types, and the HBV infected subjects in Group A and C and the HBV infected subjects in Group B in non-active phase and immunotolerance phase had no history of using liver-protective drugs, enzyme-decreasing drugs, immunomodulators or antiviral drugs. The HBV-infected subjects in Group B in active phase were not associated with a history of less than half a year of using immunomodulators or antiviral drugs. The serum specimens were kept at -20°C for test, and EDTA-K2-anticoagulated venous blood specimens were assayed in 2 h. Anti-human IgG, IgA and IgM monoclonal antibody labeled plates were purchased from Sun Biomedical Technology Co., Ltd, Beijing. Biotinylated mouse anti-human C3 antibody labeled plate was purchased from Adlitteram Diagnostic Laboratories, Inc., USA. HRP- anti-Hbs (polyclonal antibody) and ELISA HBV M reagents were provided by Shanghai Kehua Biotechnology Co., Ltd. Lymphocyte subsets reagents were purchased from Immunotech Company, France and the others included USA Abbott Axsym immunology analyzer and the auxiliary HBV M reagents, USA Bio-Rad 550 ELISA reader, and EPICS-XL flow cytometry of Coulter Company, USA.

Assaying of serum TCIC

The operation procedures were according to the methods described in the reference (Tsai et al., 1998). Serum absorbance A value (x ±2s) of health examination patients with negative HBV M was taken as a cutoff value to judge positive cases and the A values (x±s) of positive results were recorded.

Assaying of lymphocyte subsets

100 ul of EDTA-K2-anticoagulated peripheral blood was added into 10 ul CD3-FITC/CD16+56-PE/CD19-PC5, CD4-FITC/CD8-PE/CD3-PE-Cy5 and homeotype control antibody, respectively, and then they were incubated and haemolyzed. After being rinsed, the percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD19^+$ and $CD16+56^+$ were assayed on flow cytometry by a professional staff, and $CD4^+/CD8^+$ ratio was consequently obtained by calculating according to the ratio of $CD3^+CD4^+$ and $CD3^+CD8^+$.

Statistical analysis

The comparisons on ages among Group C and each phase of Group A and B were tested by median ^{2} test, and the comparisons on gender, serum TCIC positive rate, A value and lymphocyte subsets were tested by ^{2} test or Fisher's exact probability test and t test. All the data were processed by SPSS12.01 software.

RESULTS

Clinical features

Among these 44 cases of low-level HBsAg in Group A, there were 28 males and 16 females, aged from 18 to 65 years, with a mean age of 37 years. There were significant differences of age among Group B in immunotolerance phase (6 males and 4 females, 3 - 28 years, with a mean age 4 years), Group A in non-active phase, Group B in non-active phase and in washout period, and Group C (^{2} = 4.06 - 4.62, P = 0.03 - 0.04), while there was no significant difference of age or gender among other groups and phases (^{2} = 0.2 - 2.11, P = 0.15 - 0.66). 90.9% of patients with low-level HBsAg (40/44) were in non-active phase and presented low-level for 9 months to 8 years, with an average of 1.8 years. Among the other 4 patients who were not in non-active phase, 2 were immunotolerance patients and their HBV DNA were as high as 10^8 and 10^4 copies/ml, respectively; 2 were immuno-active patients, and their HBV DNA were as high as 10^6 and 10^3 copies/ml, respectively, and their ALT were between 50 and 70 U/ml. Among these 40 non-active patients, 2 patients' HBsAg/anti-HBc was positive and 2 patients' HBV DNA was as high as 10^3 copies/ml. No family aggregation or occupational difference related with low-level HBsAg was observed.

Assaying of serum TCIC and peripheral blood lymphocyte subsets

The assaying results of the serum TCIC and peripheral blood lymphocyte subsets of the three groups were shown in Tables 1 and 2.

DISCUSSION

In this study, chronic HBV-infected patients were grouped and studied on the basis of their HBsAg level and natural histories and their serum TCIC concentrations were assayed. The results revealed that 90.9% individuals of Group A (40/44) were in non-active phase and their TCIC positive rate and A value were both significantly lower than those of Group B (Table 1) (P<0.01-0.05), indicating

Table 1. Assaying results of TCIC in three groups.

Group	n	HBsAg/IgG-CIC	HBsAg/IgA-CIC	HBsAg/IgM-CIC	HBsAg/C3-CIC
A	44	7[a](0.47±0.23)[b]	3[a](0.30±0.03)[b]	4[a](0.40±0.06)[b]	2[a](0.24±0.01)[b]
Non-active phase	40	5[c](0.46±0.25)[d]	2(0.31±0.04)	3(0.38±0.06)	1[a](0.25±0)
Immune active phase	2	1(0.62±0)	1(0.30±0)	0(-)	1(0.23±0)
Immune tolerance phase	2	1(0.43±0)	0(-)	1(0.45±0)	0(-)
B	82	34(0.90±0.37)	27(0.69±0.33)	29(0.83±0.27)	38(0.63±0.22)
Non-active phase	35	13(0.80±0.34)	8(0.52±0.28)	6(0.63±0.21)	11(0.48±0.16)
Immune active phase	37	18(1.01±0.38)	15(0.86±0.31)	16(0.92±0.24)	19(0.78±0.19)
Immune tolerance phase	10	3(0.64±0.25)	4(0.43±0.16)	7(0.75±0.31)	8(0.46±0.09)
C	22	0(-)	0(-)	0(-)	0(-)

( ) absorbance (x±s); [a], P<0.01 vs B group; [b], P<0.01 vs B group; [c], P<0.05 vs non-active phase in B group; [d], P<0.05 vs non-active phase in B group.

Table 2. Assaying results of peripheral blood lymphocyte subsets in three groups(x±s).

Group	n	$CD3^+$	$CD3^-CD19^+$	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD4^+/CD8^+$	$CD16+56^+$
A							
Non-active phase	40	70.1±6.2	11.7±2.9	40.3±4.8	25.3±3.4[a]	1.6±0.2[b]	18.2±3.1
Immune active phase	2	68.3±2.1	13.3±0.6	37.2±1.8	29.4±0.9	1.3±0.1	14.3±0.4
Immune tolerance phase	2	70.8±2.3	12.1±0.5	41.3±1.5	28.2±0.7	1.5±0.1	17.6±0.3
B							
Non-active phase	35	70.2±6.3	12.1±2.6	38.1±4.7	27.3±3.6	1.4±0.3[d]	17.6±3.2
Immune active phase	37	67.7±6.1[c]	15.2±3.2[d]	36.1±3.8[d]	31.1±3.5[d]	1.2±0.4[d]	14.1±3.9[d]
Immune tolerance phase	10	73.5±6.4	12.8±2.7	41.8±4.5	29.5±4.2[c]	1.4±0.3[d]	13.6±3.1[d]
C	22	72.1±6.4	11.3±2.5	41.5±3.4	26.0±3.1	1.6±0.2	18.6±4.6

[a], P<0.05 vs non-active phase in B group; [b], P<0.01 vs non-active phase in B group; [c], P<0.05 vs C group; [d], P<0.01 vs C group.

that patients with low-level HBsAg were associated with low capability of TCIC synthesis and elimination.

TCIC, which is a special product of complicated immune response of body, is the reflection of the immune state of body (Tsai et al., 1998). The synthesis of TCIC is not only a protective reaction to eliminate harmful antigens (such as in acute hepatitis and in acute self-limited hepatitis), but also an etiological factor (such as in chronic active hepatitis), which is the pathological molecular basis of hepatic injury and chronic type B hepatitis under certain conditions (Huo et al., 1998). Besides, TCIC has heterogeneity and different patho-physiological significances in different types of HBV infection (Michelin et al., 2002). The results of this study showed that there was not only heterogeneities of TCIC positive rates and A values in Group A and Group B, but also between Group A and Group B (Table 1), which might result from the multiple inter-transition of non-active phase, immuno-active phase and immunotolerance phase in Group B, while long-term of being in non-active phase or rare inter-transition occurred in Group A.

It was discovered by assaying the lymphocyte subsets of these 3 groups that: $CD3^+CD8^+$ in non-active phase of Group A was significantly lower than that of Group B in non-active phase (P<0.05), while it was opposite as for $CD4^+/CD8^+$ (P<0.01). There was no significant difference of each lymphocyte subset between non-active phase of Group A and Group C, while there was significant difference of part or all of the lymphocyte subsets between Group B in each phase and Group C (P<0.01-0.05). The results suggested that immunotolerance phenomenon also existed in non-active phase of Group A, and should be low- dose tolerance (induced by low-level HBsAg) (Lazizi et al., 1997), as considered by the author; while the immunotolerance phenomenon existing in Group B (Salazar et al., 1995) should be high- dose tolerance (induced by high-level HBsAg). And as for non-active phase and immuno-active phase of Group B, there

was immune dysfunction phenomenon.

In conclusion, patients with low-level HBsAg were special patients during HBV infection and transmission. It was considered that the appearance and long- term existing of low-level HBsAg were not only associated with low capability of TCIC synthesis and elimination, but also associated with total or partial immunotolerance of lymphocytes (especially T and B lymphocytes) induced by low-level HBV antigen (low dose- induced immunotolerance) (Lazizi et al., 1997), and might be also associated with the molecular biology mechanism of HBV (unpublished). Thus, there was of great significance to study the mechanism of immunotolerance and the effects of interrupted immunotolerance on HBV infection, transmission, elimination and prevention in patients with low-level HbsAg (Schirmbeck et al., 2003).

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