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The Effects of Cytokines on Spontaneous Hepatitis B Surface Antigen Seroconversion in Chronic Hepatitis B Virus Infection

Jia-Feng Wu,* Hong-Yuan Hsu,* Yu-Chun Chiu,* Huey-Ling Chen,*† Yen-Hsuan Ni,*‡ and Mei-Hwei Chang*†

We examined the role of human cytokines in the natural course of hepatitis B surface Ag (HBsAg) seroconversion in chronic hepatitis B virus (HBV) infection. The clinical course of spontaneous HBsAg seroconversion was assessed in 296 chronically HBV-infected patients. Single nucleotide polymorphisms (SNPs) in IL-1β, IL-2, IL-4, IL-10, IL-12β, IL-13, IL-27, and IFN-γ genes were examined in 296 chronically HBV-infected patients and another 193 HBV recoverers. The HBsAg a determinant sequence of chronically HBV-infected subjects with and without HBsAg seroconversion was also analyzed. The start of the immune-clearance phase (serum alanine aminotransferase levels > 30 IU/l) before the age of 48 mo and hepatitis B e Ag (HBeAg) seroconversion before the age of 10 y predicted spontaneous HBsAg seroconversion in chronically HBV-infected patients (odds ratios 17.7 and 5.0; p < 0.001 and p < 0.002, respectively). The A-allele of IL-10 SNP rs1800872 was associated with higher IL-10 serum levels, and the G-allele of IL-12β SNP rs3212217 was associated with sustained high serum IL-12p70 levels during the immune-clearance phase. Both were predictors of spontaneous HBsAg seroconversion and HBV recovery (odds ratios 4.0 and 26.3; p = 0.002 and p < 0.001, respectively). Spontaneous HBsAg seroconversion was not related to sex, HBV genotype, or HBsAg a determinant mutation. The start of immune-clearance phase, age at HBeAg seroconversion, and serum IL-10 and IL-12 levels are associated with the course of the immune-clearance phase in chronic HBV infection, and are predictive of spontaneous HBsAg seroconversion and HBV recovery. The Journal of Immunology, 2015, 194: 000–000.

Chronic hepatitis B virus (HBV) infection remains a global health issue (1, 2). During the course of chronic HBV infection, hepatitis B surface Ag (HBsAg) seroconversion indicates the suppression of viral replication, whereas the HBsAg seroconversion to anti-surface Ag Ab (anti-HBs) indicates the clearance of HBV infection.

The spontaneous annual HBsAg seroconversion rate in chronically HBV-infected patients is ∼0.45–2.38% worldwide (3–7), and variation in the HLA is reportedly associated with the HBsAg seroconversion rate (8–10). A recent report showed that chronic HBV carriers with HBsAg seroconversion tend to have higher HBV viremia than those with HBsAg seroconversion (11). Hence HBsAg seroconversion and HBV recovery are the goals of chronic HBV infection treatment. Understanding the mechanism of spontaneous HBsAg seroconversion will facilitate future development of better strategies to achieve this goal. However, the long-term natural history of spontaneous HBsAg seroconversion and the relevant host genetic factors in HBV-infected patients remain largely unclear.

HBsAg a determinant is the main epitope of anti-HBs and is the main target of the humoral immune response during the course of HBsAg seroconversion. Our previous follow-up study indicated that alanine aminotransferase (ALT) levels >30 IU/l served as a cutoff for the immune-clearance phase in chronically HBV-infected patients (12). Various proinflammatory and regulatory cytokines, including IFN-γ, IL-10, and IL-12, have been reported to alter the clinical course of chronic HBV infection, but their roles in HBsAg seroconversion and HBV recovery are incompletely understood (13–16). In this study, we examined the natural course of spontaneous HBsAg seroconversion in chronically HBV-infected patients followed from early childhood to adulthood and the relationship between cytokine gene polymorphisms and cytokine phenotypes during chronic HBV infection. The impact of HBsAg a determinant mutants on spontaneous HBsAg seroconversion was also assessed.

Materials and Methods

Patients

From 1984 to 2014, we collected genomic DNA from the peripheral blood of 296 consensual chronically HBV-infected patients (123 young females) regularly followed up at the Department of Pediatrics of National Taiwan University Hospital. All had carried HBsAg for >6 mo during follow-up and met the criteria for chronic HBV infection. The patients were enrolled in a long-term follow-up program from a mean age of 8.40 y (SD 3.91 y) and were followed for 23.83 y (SD 3.60 y). Among the chronic HBV-infected cohort, 191 (64.5%) were born to HBsAg+ mothers, 78 (26.4%) were born to HBsAg mothers, and the maternal HBsAg status was unclear in 28 (9.1%) subjects. We had a total of 7053 person-years of follow-up for this cohort. Each chronic HBV-infected patient was examined at 6-mo intervals or more frequently in cases of increased ALT levels. No antiviral agent was given during the follow-up period.

In this study, clinical phases of chronic HBV infection were defined as: 1) immune-tolerance phase: HBeAg+ and its Ab (anti-HBe)-negative with ALT <30 IU/l; (12) 2) immune-clearance phase: HBeAg+, anti-HBe with...
ALT >30 IU/l (12); 3) post-HBeAg seroconversion phase: HBeAg+, anti-HBs+ for >6 mo, ALT <30 IU/l; and 4) HBsAg seroconversion was defined as serum HBsAg clearance and the presence of anti-HBs Abs for >6 mo.

Chronically HBV-infected subjects observed to have HBsAg seroconverted to anti-HBs for >6 mo were defined as spontaneous HBsAg seroconversion. The remaining chronically HBV-infected subjects were defined as HBsAg nonconverters.

We enrolled 193 age-matched subjects (72 males, 121 females; 34.3 ± 13.0 y) from a community HBV cross-sectional screening project in Taipei City to serve as the HBV recovery group. The subjects were HBsAg+, anti-HBs+, and HBV core Ab (anti-HBc)-positive were considered as recovered from either acute or chronic HBV infection. The HBV recovery group was pooled with the HBsAg seroconverters from chronically HBV-infected subjects and termed the HBV seroconversion/recovery group, for the comparison of differences between the subjects with persistent HBV infection and those who cleared the virus after acute or chronic infection.

The Institutional Review Board and Ethics Committee of the hospital approved the study protocol. All patients and/or their parents/guardians provided written informed consent.

**HBV serological tests, viral load, and HBV genotyping**

Serum samples were obtained during each visit for HBV marker and liver function profiles. HBV markers, including HBsAg, anti-HBs, HBcAg and anti-HBc, and anti-HBe were assessed (Abbott Laboratories, North Chicago, IL). Quantitative HBsAg titters were checked using the Architect HBsAg QT (Abbott Laboratories). The HBV genotypes and viral load of each patient were determined using real-time PCR and melting temperature curve analysis system (17). The serum samples of 248 (83.78%) subjects in the immune-tolerance phase before HBsAg seroconversion were available for HBV viral load determination.

**DNA extraction and single nucleotide polymorphism genotyping**

Genomic DNA was extracted from venous blood of each participant using the Puregene kit (Gentra Systems, Minneapolis, MN) following the manufacturer’s instructions. Thirteen single nucleotide polymorphisms (SNPs) in eight cytokine genes (IL-1β, IL-2, IL-4, IL-10, IL-12p40, IL-13, IL-27, and IFN-γ) were identified using GenBank and a Japanese and Han Chinese SNP database. We followed the SNP genotyping procedures from our previous study (15).

**PBMC isolation and in vitro culture**

Whole-blood samples were collected from 67 chronically HBV-infected subjects with spontaneous HBsAg seroconversion during the study period for PBMC isolation and in vitro study. PBMC was isolated by centrifugation on Ficoll-Hypaque density medium (Pharmacia, Milton Keynes, UK) and washed three times before resuspension in a culture medium of RPMI 1640, t-glutamine, penicillin, streptomycin, and 10% FCS at a density of 2 × 10⁶ cells/ml in a microculture plate. PBMCs (1 × 10⁶ cells/ml) were incubated in recombinant HBcAg (1 μg/ml) for the PBMC stimulation cytokine release assay. The plates were incubated in a humidified incubator with 5% CO₂ at a temperature of 37°C. The supernatant was collected on the sixth day of culture for ELISA.

**ELISA determination of serum IL-10 and IL-12 levels**

Sequential serum samples available for 116 (39.2%) chronically HBV-infected patients were assessed for serum cytokine levels. The sequential conditions were: 1) the immune-tolerance phase, 2) the immune-clearance phase, and 3) the post-HBeAg seroconversion phase. These serum samples were assessed for serum IL-10, IL-12p70, and IL-12p40 levels using a sensitive ELISA technique (Duoset; R&D Systems, Minneapolis, MN). The supernatants of PBMC cultures were also assessed for IL-10, IL-12p70, and IL-12p40 concentrations using the ELISA.

**Furin knockdown in HepG2.2.15 cells**

HepG2.2.15 cells (HBV replication-competent human hepatoma cell line) were gifts from Dr. H.L. Wu of National Taiwan University Hospital. Furin knockdown by RNA interference was used to assess the impact of furin on biosynthesis of HBV viral particles in HepG2.2.15 cells (1 × 10⁵ cells in six-well culture plates) in anti-MEM medium (Thermo Scientific) with 10% heat-inactivated FBS. One small interfering RNA (SR303336A-GGACAAUGAGAUAUGUAGAGGUTT) specific to furin (OriGene Technologies, Rockville, MD) was used to knock down furin. Transfection was performed using TurboFect (Fermentas, MD), according to the manufacturer’s instructions.

**HBsAg a determinant mutant analysis in serum before HBeAg seroconversion**

Serum samples from subjects with and without spontaneous HBsAg seroconversion before HBsAg seroconversion were collected for sequence analysis of HBsAg a determinant (aa 110–160). We followed the procedures from our previous study (18).

**Statistical analysis**

The STATA (StataCorp, College Station, TX) and MedCalc (version 12.4.0; MedCalc Software, Ostend, Belgium) software packages were used for statistical analyses. Student t test with unequal variance or Mann–Whitney U tests was used for continuous variables to test the differences in 95% confidence interval (CI)/mean or distribution/median between the two groups. Fisher’s exact test or χ² tests were used to test differences in incidence between groups. Receiver operating characteristic (ROC) curve analysis was used to determine the cutoff. Univariate and multivariate logistic regression analysis were also used to test the odds ratio (OR), 95% CI, and p values between differing SNP genotypes. Life table analysis was also used for the cumulative spontaneous HBsAg seroconversion rate.

### Table I. General characteristics of 296 chronic HBV-infected patients with and without spontaneous HBsAg seroconversion

<table>
<thead>
<tr>
<th></th>
<th>HBsAg Nonconverter (n = 280)</th>
<th>HBsAg Seroconverter (n = 16)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial HBsAg titer, mean ± SD, log₁₀</td>
<td>4.09 ± 1.10</td>
<td>3.39 ± 2.28</td>
<td>0.02</td>
</tr>
<tr>
<td>HBV viral load at immune-tolerance phase, log₁₀ copies/ml³</td>
<td>8.10 ± 1.11</td>
<td>6.96 ± 2.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Initial ALT levels, mean ± SD, IU/l</td>
<td>37.34 ± 75.77</td>
<td>178.38 ± 245.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>First ALT &gt; 30 IU/l age, mean ± SD, y</td>
<td>15.37 ± 8.41</td>
<td>6.16 ± 5.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak ALT levels during immune-clearance phase, mean ± SD, IU/l</td>
<td>170.41 ± 12.52</td>
<td>257.94 ± 61.35</td>
<td>0.04</td>
</tr>
<tr>
<td>Final follow-up age, mean ± SD, y</td>
<td>32.23 ± 5.26</td>
<td>32.14 ± 5.92</td>
<td>0.95</td>
</tr>
<tr>
<td>HBsAg titer drop rate per year, mean ± SD, log₁₀/10 IU/ml</td>
<td>0.08 ± 0.10</td>
<td>0.32 ± 0.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HBsAg seroconversion age, mean ± SD, y</td>
<td>16.58 ± 8.69</td>
<td>10.98 ± 5.73</td>
<td>0.01</td>
</tr>
<tr>
<td>(222 versus 16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>160 (57.1%)</td>
<td>3 (18.8%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>120 (42.9%)</td>
<td>13 (81.2%)</td>
<td>0.06</td>
</tr>
<tr>
<td>HBsAg seroconversion, n (%)</td>
<td>203 (72.5%)</td>
<td>16 (100%)</td>
<td>0.02</td>
</tr>
<tr>
<td>HBV genotype, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype B</td>
<td>212 (75.7%)</td>
<td>12 (75%)</td>
<td></td>
</tr>
<tr>
<td>Genotype C</td>
<td>53 (18.9%)</td>
<td>4 (25%)</td>
<td></td>
</tr>
<tr>
<td>Mixed genotypes B and C</td>
<td>15 (5.4%)</td>
<td>0 (0%)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

³HBV viral load at immune-tolerance before HBsAg seroconversion is available in 233 HBsAg nonconverters and 15 HBsAg seroconverters for comparison.
Cox proportional hazards model and Kaplan–Meier analysis were applied for the survival analyses. All SNPs were assessed for Hardy–Weinberg equilibrium using the χ² test. Linkage disequilibrium was also assessed in all pairings of the 13 SNPs. A p value <0.05 was considered to indicate statistical significance in single comparisons. The Bonferroni correction was used to adjust p values for multiple comparisons.

Results

Natural course of spontaneous HBsAg-seroconversion in chronically HBV-infected patients

Among the chronically HBV-infected study subjects (n = 296), 16 developed spontaneous HBsAg seroconversion during the follow-up. The median spontaneous HBsAg seroconversion age was 13.50 y (range, 4.11–32.50 y), and the median interval between HBsAg seroclearance and HBsAg seroconversion was 0.70 y (range, 0–8.11 y) in spontaneous HBsAg seroconverters. The overall cumulative incidence of spontaneous HBsAg seroconversion was 5.40%, whereas the annual HBsAg seroconversion rate was 0.25% in chronic HBV-infected subjects. The age-specific annual spontaneous HBsAg seroconversion rates were 0.54, 0.18, 0.03, and 0.07% person-years for subjects <10, 10–19, 20–29, and >30 y of age, respectively, in chronically HBV-infected subjects.

Chronically HBV-infected subjects with spontaneous HBsAg seroconversion were demonstrated to have serum ALT levels >30 IU/l earlier than those without HBsAg seroconversion (p < 0.001; Table I). The ROC curve analysis yielded the start of immune clearance (first ALT > 30 IU/l age) cutoff at 48 mo of age for optimum prediction of spontaneous HBsAg seroconversion (sensitivity 62.5%, specificity 93.2%, area under curve 82.3%; p < 0.001). The start of immune clearance in subjects <48 mo of age was associated with a higher HBsAg seroconversion rate (OR 17.66, 95% CI 5.93–52.64; p < 0.001); this phenomenon was consistent in the survival analysis (hazard ratio [HR] = 29.24, p < 0.001; Fig. 1, Supplementary Tables I, II).

The HBeAg seroconversion age in this cohort was also younger in spontaneous HBsAg seroconverters (Table I). The ROC curve analysis yielded the start of immune clearance (first ALT > 30 IU/l age) cutoff at 10 y for the best prediction of spontaneous HBsAg seroconversion (sensitivity 56.3%, specificity 77.5%, area under the curve 69.1%; p = 0.003). HBeAg seroconversion at <10 y of age was associated with higher HBsAg seroconversion rate (OR 5.03; 95% CI 1.80–14.08; p = 0.002); this phenomenon was also consistent in the survival analysis (HR 5.78; p = 0.001; Fig. 1, Supplemental Table II).

In multivariate analysis, the occurrence of immune clearance at <48 mo of age and HBeAg seroconversion at <10 y of age predicted earlier HBsAg seroconversion (HRs 23.38 and 3.55; p < 0.001 and p = 0.02, respectively; Supplemental Table II). The start of immune clearance at <48 mo of age was a predictor of HBeAg seroconversion at <10 y of age (OR 5.81; 95% CI 2.59–13.06; p < 0.001).

Spontaneous HBsAg seroconverters also had greater peak ALT levels during the immune-clearance phase, lower initial HBsAg titers, greater overall annual rates of decreases in HBsAg titers, lower HBV viral loads in the immune-tolerance phase, and higher HBeAg seroconversion rates (Table I). There was no significant relationship between HBV genotypes and HBsAg seroconversion (p = 0.57).

In spontaneous HBsAg seroconverters (n = 16), the median age at which serum ALT levels reached >30 IU/l was 2.58 y (interquartile range [IRQ], 1.98–10.51 y). The median duration of the immune-clearance phase with serum ALT >30 IU/l was 1.44 y (IRQ, 0.87–7.85 y), and the median interval from the normalization of ALT (≤30 IU/l) to the HBsAg seroconversion was 4.46 y (IRQ, 1.98–10.51 y). The median ALT level 6 mo before the occurrence of HBsAg seroconversion was 17 IU/l (IRQ, 14–23 IU/l) in spontaneous HBsAg seroconverters.

Subgroup analysis in subjects with HBV viral load data at immune-tolerance phase

In subjects with available HBV viral load data in the immune-tolerance phase (n = 248), those with an initial baseline HBV viral load <8 log₁₀ copies/ml had a higher HBsAg seroconversion rate (OR 3.42; 95% CI 1.13–10.33; p = 0.03) in single logistic regression analysis. However, an initial HBV viral load <8 log₁₀ copies/ml was not significant (OR 1.48; 95% CI 0.40–5.48; p = 0.56) in a multiple logistic regression analysis considering the start of immune clearance before 48 mo of age and HBeAg seroconversion before 10 y of age. In contrast, the start of immune clearance before 48 mo of age (OR 12.15; 95% CI 3.32–44.41; p < 0.001) and HBeAg seroconversion before 10 y of age (OR 4.38; 95% CI 1.23–15.53; p = 0.02) remained significant in the subgroup multivariate analysis.

Cytokine SNPs and HBsAg seroconversion/HBV recovery

The IL-10 SNP rs1800872, IL-12B SNP rs35212217, and IL-13 SNP rs1800925 were statistically significant with p < 0.004 after Bonferroni correction (Supplemental Table III). The prevalence rate of IL-10 SNP rs1800872 A allele-carrying subjects in the HBV recovery group (97.4%) was greater than the HBsAg nonconverter
group (90%; \( p = 0.002 \)), but there was no difference compared with the HBsAg-seroconversion group (93.8%; \( p = 0.38 \)). The prevalence rate of \( IL-12 \) SNP rs3212217 \( G \) allele-carrying subjects was greater in the HBV recovery group than the HBsAg nonconverter group (100% versus 77.9%; \( p < 0.0001 \)), but different from the HBsAg seroconversion group (87.5%; \( p > 0.004 \)). The prevalence rate of \( T \) allele-carrying subjects at \( IL-13 \) SNP rs1800925 was also higher in the HBV recovery group (50.8%) than the HBsAg non-converter group (29.3%; \( p < 0.001 \)), but was not different from the HBsAg seroconversion group (37.5%; \( p = 0.31 \)). Because the prevalence of these three SNP genotypes did not differ between the HBV recovery and HBsAg seroconversion groups, these two groups were pooled in the following analysis.

The positive predictive values of HBsAg seroconversion/HBV recovery were 97.13, 99.04, and 49.76%, respectively, for \( A/A \), \( A/C \), and \( C/C \) genotype carriers (Table III). Subjects with \( A \)-allele at \( IL-10 \) SNP rs1800872 had greater overall annual rates of decreases in HBsAg titers (0.09 log \( 10 \) IU/ml per year; \( p = 0.0001 \)) and greater peak ALT levels during the immune-clearance phase than \( C/C \) genotype-carrying subjects (212.79 ± 17.72 versus 140.82 ± 20.34 IU/l; \( p = 0.009 \)). There was no significant relation among \( IL-13 \) SNP rs1800925 genotypes in terms of ALT levels or HBsAg titers. There were also no significant differences in the prevalence of \( IL-12B \) (rs1143634, rs1143627, rs169444), \( IL-2 \) (rs2069762), \( IL-4 \) (rs2243250), \( IL-10 \) (rs3024495, rs1800871, rs1800872, rs3024491), \( IL-27 \) (rs181206), or \( INF-\gamma \) (rs2069727) SNPs among these three groups (\( p > 0.004 \); Supplemental Table III).

**Table II.** Logistic regression analysis to identify significant cytokine markers of HBsAg seroconversion and HBV recovery

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Genotype</th>
<th>OR (95% CI)</th>
<th>( p )</th>
<th>OR (95% CI)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-10</strong></td>
<td>rs1800872</td>
<td>A/A+ A/C versus C/C</td>
<td>3.76 (1.53–9.25)</td>
<td>0.004</td>
<td>4.47 (1.76–11.36)</td>
</tr>
<tr>
<td><strong>IL-12B</strong></td>
<td>rs3212217</td>
<td>G/G+ C/G versus C/C</td>
<td>29.44 (7.11–121.88)</td>
<td>&lt;0.001</td>
<td>30.74 (7.36–128.23)</td>
</tr>
<tr>
<td><strong>IL-13</strong></td>
<td>rs1800925</td>
<td>T/T+C/T versus C/C</td>
<td>2.39 (1.65–3.48)</td>
<td>&lt;0.001</td>
<td>2.45 (1.64–3.66)</td>
</tr>
</tbody>
</table>

Univariate and multivariate logistic regression were applied to test the OR, 95% CI, and \( p \) value of identified cytokine genotypes on the prediction of HBsAg-seroconversion and recovery.

*The \( p \) value was adjusted to 0.016 by Bonferroni correction for multiple comparisons.*

Subjects with the \( A \)-allele at \( IL-10 \) SNP rs1800872, which was associated with higher HBsAg seroconversion/HBV recovery, exhibited greater serum IL-10 levels during the course of chronic HBV infection than \( C/C \) genotype-carrying subjects (Table IV). The ratio of IL-12p70 during the immune-clearance phase compared with the immune-tolerance phase was greater in \( G \) allele- than \( C/C \) genotype-carrying subjects (\( p = 0.04 \)), indicating a more sustained proinflammatory effect in \( IL-12B \) SNP rs3212217 \( G \) allele-carrying subjects during the immune-clearance phase (Table V). The ratio of serum IL-12p70/IL-12p40 during the immune-clearance phase was also borderline greater in \( G \) allele- than \( C/C \) genotype-carrying subjects (\( p = 0.07 \), which indicated a greater proinflammatory effect in the immune-clearance phase in \( G \) allele-carrying subjects. The in vitro PBMC culture study showed that PBMCs isolated from \( IL-10 \) SNP rs1800872 \( A \)-allele carrying subjects (\( n = 60 \)) secreted more IL-10 than \( C/C \) genotype-carrying subjects (\( n = 7 \); 683.45 ± 117.49 versus 270.11 ± 123.19 pg/ml; \( p = 0.02 \)). The PBMC supernatant IL-12p70/IL-12p40 ratio was also greater in \( IL-12B \) SNP rs3212217 \( G \) allele-carrying subjects (\( n = 59 \)) than \( C/C \) genotype-carrying subjects (\( n = 8 \); 1.14 ± 0.13 versus 0.58 ± 0.22; \( p = 0.04 \)). The knockdown of furin by small interfering RNA in HepG2.2.15 hepatoma cells suppressed the biosynthesis of HBeAg, HBsAg, and HBV viral load (Supplemental Table IV).

**Table III.** Overall HBsAg titer drop rates according to cytokine genotype in chronic HBV-infected subjects (\( n = 296 \))

<table>
<thead>
<tr>
<th>HBsAg Titer Drop Rate (log(_{10}) IU/ml)</th>
<th>Mean ± SE</th>
<th>95% CI</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-10</strong> rs1800872</td>
<td>A/A and A/C genotype (( n = 267 ))</td>
<td>0.09 ± 0.01</td>
<td>0.08–0.11</td>
</tr>
<tr>
<td>C/C genotype (( n = 29 ))</td>
<td>0.05 ± 0.09</td>
<td>0.02–0.09</td>
<td></td>
</tr>
<tr>
<td><strong>IL-12B</strong> rs3212217</td>
<td>G/G and C/G genotype (( n = 232 ))</td>
<td>0.10 ± 0.01</td>
<td>0.08–0.12</td>
</tr>
<tr>
<td>C/C genotype (( n = 64 ))</td>
<td>0.07 ± 0.01</td>
<td>0.05–0.10</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>IL-13</strong> rs1800925</td>
<td>T/T and C/T genotypes (( n = 88 ))</td>
<td>0.09 ± 0.01</td>
<td>0.07–0.11</td>
</tr>
<tr>
<td>C/C genotype (( n = 208 ))</td>
<td>0.09 ± 0.01</td>
<td>0.06–0.11</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Student \( t \) test with unequal variance was used to test differences in the 95% CI/mean of overall HBsAg titer drop rate in log\(_{10}\) ratio between the two genotype groups.
such as treatment age and drug design. This observation may facilitate formulation of improved therapeutic policies, particularly in HBV-infected patients and subsequent HBsAg seroconversion during the immune mechanisms that promote early immune clearance. We further analyzed the HBsAg 10 SNP rs1800872 and A/G than those without HBsAg seroconversion. Both the HBsAg titers, and higher annual rates of decreases in HBsAg titers during the immune-clearance phase, lower HBV viremia have a greater HCC risk than HBV-naive subjects (20, 21). Thus, HBsAg seroconverters with PCR− HBV DNA and/or a history of chronic infection should receive medical attention and undergo regular HCC screening.

Table IV. Relation between IL-10 rs1800872 genotype and IL-10 serum levels in chronic HBV-infected patients during the course of chronic HBV infection

<table>
<thead>
<tr>
<th>IL-10 Serum Levels in Chronic HBsAg Carriers</th>
<th>A/A and A/C Genotypes (n = 105)</th>
<th>C/C Genotype (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>95% CI</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Immune tolerance phase, pg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>581.65 ± 108.48</td>
<td>366.53–796.78</td>
</tr>
<tr>
<td>Immune clearance phase, pg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>488.81 ± 89.66</td>
<td>310.07–667.55</td>
</tr>
<tr>
<td>Post-HBeAg seroconversion phase, pg/ml&lt;sup&gt;c&lt;/sup&gt;</td>
<td>515.26 ± 83.74</td>
<td>349.19–681.32</td>
</tr>
</tbody>
</table>

Student t test with unequal variance was used to test differences in the 95% CI/mean of serum IL-10 levels between the two genotype groups.

<sup>a</sup>Immune-tolerance phase: ALT < 30 IU/l, HBeAg(+), anti-HBe(−).
<sup>b</sup>Immune-clearance phase: ALT > 30 IU/l, HBeAg(+), Anti-HBe(−).
<sup>c</sup>Post-HBeAg seroconversion phase: ALT < 30 IU/l, HBeAg(−), anti-HBe(+).

Table V. Relation between IL-12β rs3212217 genotype and IL-12 (IL-12) serum levels in chronic HBV-infected patients during the course of chronic HBV infection

<table>
<thead>
<tr>
<th>IL-12p70 serum levels, pg/ml</th>
<th>G/G and C/G Genotypes (n = 95)</th>
<th>C/C Genotype (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>95% CI</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Immune-tolerance phase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2242.97 ± 377.31</td>
<td>1493.81–2929.13</td>
</tr>
<tr>
<td>Immune-clearance phase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2155.04 ± 411.82</td>
<td>1333.04–2977.04</td>
</tr>
<tr>
<td>Post-HBeAg seroconversion phase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2485.19 ± 423.94</td>
<td>1643.44–3326.90</td>
</tr>
<tr>
<td>Ratio of IL-12p70 level during immune clearance/tolerance phases (times)</td>
<td>3.25 ± 1.24</td>
<td>0.77–5.73</td>
</tr>
</tbody>
</table>

Student t test with unequal variance was used to test differences in the 95% CI/mean of serum IL-12 levels between the two genotype groups.

<sup>a</sup>Immune-tolerance phase: ALT < 30 IU/l, HBeAg(+), anti-HBe(−).
<sup>b</sup>Immune-clearance phase: ALT > 30 IU/l, HBeAg(+), Anti-HBe(−).
<sup>c</sup>Post-HBeAg seroconversion phase: ALT < 30 IU/l, HBeAg(−), anti-HBe(+).

No difference in HBsAg a determinant mutant
We further analyzed the HBsAg a determinant sequence (aa 110–160) in 12 spontaneous HBsAg seroconverters and 48 age-, sex-, and follow-up period-matched HBsAg nonconverters during the immune-clearance phase in this cohort study. Ten (83.33%) of the spontaneous HBsAg seroconverters and 29 (60.42%) of the HBsAg nonconverters carried a wild-type HBsAg a determinant sequence; the difference was not significant (p = 0.19).

Discussion
In this study, we demonstrated the natural course of spontaneous HBsAg seroconversion in chronically HBV-infected patients from childhood to young adulthood. Chronically HBV-infected patients with spontaneous HBsAg seroconversion had earlier entrance into the immune-clearance phase, earlier HBeAg seroconversion, greater ALT levels during the immune-clearance phase, lower HBV viral loads during the immune-tolerance phase, lower initial HBsAg titers, and higher annual rates of decreases in HBsAg titers than those without HBsAg seroconversion. Both the A allele at IL-10 SNP rs1800872 and G allele at IL-12β SNP rs3212217 predicted greater peak ALT levels during the immune-clearance phase before HBeAg seroconversion, and both were predictors of spontaneous HBsAg seroconversion/HBV recovery. Understanding the immune mechanisms that promote early immune clearance and following earlier HBeAg seroconversion in chronically HBV-infected patients and subsequent HBsAg seroconversion may facilitate formulation of improved therapeutic policies, such as treatment age and drug design.

Up to 50–60% of residents in Taiwan born before the universal HBV vaccination program were found to be HBV recoverers/ HBsAg seroconverters [HBsAg(−), anti-HBs(+), and anti-HBe(+)], which is considerably greater than the prevalence rate of acute HBV infection (2.33/100,000) reported by the Centers for Disease Control in Taiwan (19). We speculate that the difference may be mainly because of spontaneous recovery from either acute or chronic HBV infection before the individuals receive the HBV marker checkups. Hence chronically HBV-infected subjects with spontaneous HBsAg seroconversion may be regarded as having recovered from acute HBV infection. Although the hepatocellular carcinoma (HCC) risk declines significantly after HBsAg seroconversion in HBV-infected patients, HBsAg seroconverters with viremia have a greater HCC risk than HBV-naive subjects (20, 21). Thus, HBsAg seroconverters with PCR− HBV DNA and/or a history of chronic infection should receive medical attention and undergo regular HCC screening.

Evidence regarding the role of the host genetic background in different clinical outcomes of HBV infection is accumulating (9–16, 22). We found that the IL-10 SNP rs1800872 (-592/A), IL-12β SNP rs3212217 (-10993/C), and IL-13 SNP rs1800925 (-1112/C/T) predicted spontaneous HBsAg seroconversion and HBV recovery in this study. High IL-10 production genotype was shown to be associated with greater ALT levels during the immune-clearance phase and greater rate of decrease in HBsAg titer in this study, which are both key elements before spontaneous HBsAg seroconversion. A previous study of chronically HBV-infected patients reported a positive relation between serum IL-12p70 and ALT at the time of acute HBV infection (22). We speculate that the difference may be mainly because of spontaneous recovery from either acute or chronic HBV infection before the individuals receive the HBV marker checkups. Hence chronically HBV-infected subjects with spontaneous HBsAg seroconversion may be regarded as having recovered from acute HBV infection. Although the hepatocellular carcinoma (HCC) risk declines significantly after HBsAg seroconversion in HBV-infected patients, HBsAg seroconverters with viremia have a greater HCC risk than HBV-naive subjects (20, 21). Thus, HBsAg seroconverters with PCR− HBV DNA and/or a history of chronic infection should receive medical attention and undergo regular HCC screening.

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ALT and IL-10 levels during the immune-clearance phase; this is consistent with our observation (23). A Japanese study also showed that the IL-10 high-producer genotype predicts self-limiting HBV infection (24). IL-10 was reported to activate CTLs in the presence of IL-2, whereas IL-2 is downstream of IL-12 (16). Our study indicates that a high IL-10 level plays a beneficial role in spontaneous HBsAg seroconverters, probably in combination with coexisting proinflammatory and regulatory cytokines.

IL-12p70 is a 70-kDa heterodimer, consisting of a 40-kDa (p40) and a 35-kDa (p35) subunit. IL-12p70 is an important proinflammatory cytokine that promotes differentiation of Th1 cells, enhances NK cell cytotoxicity and activation of IFN-γ pathway, and rescues the antiviral function of exhausted HBV-specific T cells (25-27). IL-12p40 homodimer could antagonize the proinflammatory function of the IL-12p70, and a high serum IL-12p70/IL-12p40 ratio reflects a shift to a proinflammatory process (28). Carriers of the G allele of IL-12β SNP rs3212217, markers of spontaneous HBsAg- serum conversion and HBV recovery, had greater ALT levels during the immune-clearance phase, greater IL-12p70/IL-12p40 ratios, and sustained IL-12p70 levels during the immune-clearance phase in chronically HBV-infected patients. IL-12p70 is associated with both cytolytic and noncytolytic HBV suppression pathways, and has been investigated as an anti-HBV agent in clinical trials (16, 29, 30).

In our previous study of liver tissues from chronically HBV-infected patients, the IL-10 and IL-12 expression levels positively correlated with the expression levels of IFN-γ and negatively with those of furin during the immune-clearance phase with ALT elevation (16). We further demonstrated that knockdown of furin suppressed the biosynthesis of HBeAg, HBsAg and decreased the HBV viral load in hepatoma cells. Furin was reported to be essential for the maturation of HBeAg in human hepatocytes; therefore, the suppression of furin may lead to cytosolic accumulation of HBeAg proprotein p22 (31). Cytosolic accumulation of HBeAg proprotein p22 was also reported to decrease HBV replication because of the formation of unstable nucleocapsids (32). We speculate that elevation of ALT during the immune-clearance phase may accompany upregulation of IL-10 and IL-12 and downregulation of furin, which results in the suppression of HBV biosynthesis. Chronically HBV-infected subjects with spontaneous HBsAg seroconversion in this study had greater peak ALT levels during the immune-clearance phase at an extremely young age, and all had normalized serum ALT levels for a median of 4.5 years before spontaneous HBsAg seroconversion. These results further support the importance of prompt inflammatory responses, earlier entrance of immune clearance, and the transition from the cytolytic to the noncytolytic HBV inhibition pathway in the process of spontaneous HBsAg seroconversion in chronic HBV infection.

IL-13 SNP rs1800925 (-1121C/T) was also found to be associated with HBV recovery and spontaneous HBsAg seroconversion, albeit with a relatively minor effect, and was not associated with serum ALT levels in this study. The T allele of IL-13 SNP rs1800925 has been reported to enhance IL-13 promoter activity (33). IL-13 has also recently been shown to play an important role in NK T cell–mediated responses in HBV transgenic mice (34). IL-13 has also recently been shown to play an important role in cytolytic NK T cells (34). IL-13 has also recently been shown to play an important role in cytolytic NK T cells (34).

In conclusion, earlier entrance into the immune-clearance phase predicts subsequent spontaneous HBeAg and then HBsAg seroconversion in chronically HBV-infected patients. The A allele of IL-10 SNP rs1800872 was associated with higher serum IL-10 levels, and the G allele of IL-12β SNP rs3212217 was associated with sustained high serum IL-12p70 levels during the immune-clearance phase of chronic HBV infection. Both were associated with higher ALT levels during the immune-clearance phase and were predictors of spontaneous HBsAg seroconversion and HBV recovery.

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Disclosures
The authors have no financial conflicts of interest.

References


