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Research Article

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Interleukin-12 Induction of Th1 Cytokines Is Important for Viral Clearance in Chronic Hepatitis B

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Abstract

Interleukin-12, a cytokine with an important role against intracellular pathogens, promotes Th1 cell development, cell-mediated cytotoxicity, and interferon- γ production. We investigated the immunoregulatory role of IL-12 in 72 chronic hepatitis B virus (HBV) carriers, 33 of whom were monitored longitudinally during interferon- α treatment. Serum levels of IL-12 heterodimer, IL-12 p40 subunit, IL-4, and Th1 cytokines were determined by specific ELISAs, and hepatitis B core antigen-specific T cell response by a proliferation assay. Chronic HBV carriers had higher serum levels of IL-12 and IL-12 p40 in comparison with controls ($P < 0.01$), suggesting that IL-12 production is not impaired. The longitudinal analysis revealed a further substantial increase ($> 2.5 \times$ baseline level) of bioactive IL-12 and Th1 cytokines in patients who cleared HBV and seroconverted to anti-hepatitis B e, unlike the 23 nonresponders with persistent HBV replication ($P < 0.01$). The IL-12 peak followed the peak of hepatocytolysis by 9.8 ± 2.8 wk and occurred either before or simultaneously with hepatitis B e seroconversion. Hepatitis B core antigen-specific T cell proliferation closely correlated with hepatocytolysis and increased significantly in all patients (8 responders and 15 nonresponders) who developed hepatitis flare, irrespective of the virological outcome. These results provide in vivo evidence that IL-12 may have an important role for viral clearance in chronic HBV infection. (*J. Clin. Invest.* 1997. 99:3025–3033.) **Key words:** interleukin-12 • hepatitis B virus • chronic hepatitis B • T helper cytokines • Th1/Th2 cells

Introduction

The host immune response to hepatitis B virus (HBV)¹ is a critical factor in determining the outcome of HBV infection. Patients with self-limited, acute hepatitis B develop strong

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1. *Abbreviations used in this paper:* ALT, alanine aminotransferase; CTL, cytotoxic T lymphocyte; HAI, histological activity index; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; POD, peroxidase-conjugated antibody; SI, stimulation index.

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polyclonal HLA class I- and II-restricted T cell responses to viral peptides, while these responses are weak and narrowly focused in chronic HBV carriers (1). During the natural course of chronic HBV infection, some patients undergo a spontaneous exacerbation of the liver damage with elevation of serum aminotransferases, which may result in hepatitis B e antigen (HBeAg) to anti-HBe seroconversion and viral clearance. These hepatitis flares are associated with enhancement of the virus-specific T helper cell reactivity (2–4).

Recent studies have defined two T helper cell subsets, Th1 and Th2, which are characterized by distinct and mutually exclusive patterns of cytokine production and different functions (5). Th1 cells produce IFN- γ , IL-2, and promote cellular immune reactions, while Th2 cells produce IL-4, -5, and -10 and enhance humoral immune response. Th1/Th2 imbalances are important in the pathogenesis of chronic viral infections in humans (6). Using sensitive, unconventional immunoassays, it has been shown that asymptomatic HBV carriers with high level of virus replication are characterized by Th2 type immune reactions, while exacerbations of chronic hepatitis B are associated with activation of Th1-type responses (7, 8). Thus, preferential activation and commitment towards Th1 or Th2 cell subsets would influence the clinical consequences of HBV infection.

Interleukin-12 is the dominant cytokine, which specifically promotes Th1 cell differentiation and suppresses Th2 function (6). Biologically active IL-12 is a heterodimer, composed of two subunits of 40 (p40) and 35 (p35) kD. IL-12 stimulates the production of IFN- γ by peripheral blood mononuclear cells and augments natural killer cell cytotoxicity and antigen-specific proliferation of CD8+ cytotoxic T lymphocytes (CTL) (9, 10). Accumulating evidence suggests that this cytokine plays a central role in efficient immune response to bacterial and parasitic infections (11, 12) and can correct some of the deficient T cell responses in HIV-infected patients (13). The role of IL-12 in directing the immune responses in patients with chronic HBV infection has not been investigated.

The aim of our study was to investigate the immunoregulatory role of IL-12 for viral clearance in patients with chronic HBV infection. For this purpose, we longitudinally studied serum IL-12 levels, hepatitis B core antigen (HBcAg)-specific T cell proliferation and markers of Th1 type response, IFN- γ and IL-2, in chronic HBV carriers undergoing IFN- α treatment, which represents in a condensed time frame the process of HBV elimination. This allows a time course analysis of these immune reactions in relation to the exacerbation of hepatocytolysis and viral clearance, manifested by the alanine aminotransferase (ALT) flare and HBeAg to anti-HBe seroconversion, respectively.

Methods

Patients. The study examined 72 consecutive adults with chronic HBV infection (56 male, 16 female, aged 33.9 ± 11.6 yr) seropositive

Table I. Biochemical and Histological Profile of Patients and Controls

Liver histology HBV serology	Controls	Group 1	Group 2	Group 3
	HBsAg – ve	Minimal histologic activity HBeAg + ve HBV DNA + ve	Chronic active hepatitis HBeAg + ve HBV DNA + ve	Minimal histologic activity anti-HBe + ve HBV DNA – ve
Patient number	23	27	26	19
Age	30.8±6.9	29.4±7.3	36.2±13.6	37.3±11.8
Male/female	26/19	19/8	23/3	12/7
Histological activity index		1.8±0.6	5.46±2.3	1.6±0.3
Serum ALT (IU/ml)	21.6±6.5	32.6±14.6	131.2±99.3	24.1±5.1
Serum HBV DNA (pg/ml)		233.0±214.3	166.8±212.9	0

for hepatitis B surface antigen (HBsAg) for at least 12 mo. All patients were seronegative for markers of hepatitis C virus (HCV), hepatitis Delta virus, and HIV (Table I).

A percutaneous liver biopsy was performed in each patient as part of the diagnostic evaluation with informed consent. Liver histology was assessed blindly, with respect to serum aminotransferases and HBeAg/HBV DNA status, on formalin-fixed paraffin sections, and the severity of necroinflammatory activity in the liver was assessed semiquantitatively (14). Portal tract inflammation, periportal piecemeal necrosis, and lobular inflammation were each graded on a 0–3 scale and a histological activity index (HAI) was calculated, with a maximum value of nine.

The patients were assigned to three distinct groups according to the degree of necroinflammatory activity and the presence of HBV replication. Group 1: 27 patients with active HBV replication (HBeAg positive, anti-HBe negative, and HBV DNA positive), but only minimal histological activity in the liver biopsy (HAI 1–3), and normal or minimally elevated serum alanine aminotransferase (ALT < 1.5 × upper limit of normal; normal range: 10–50 IU/liter). Group 2: 26 patients with active HBV replication, chronic active hepatitis B (with moderate or severe necroinflammatory activity, HAI 4–9) in the liver biopsy, and elevated serum ALT (ALT > 1.5 × upper limit of normal). Group 3: 19 chronic HBsAg carriers with no serological evidence of ongoing viral replication (HBeAg negative, anti-HBe positive, and HBV DNA negative), minimal histological activity (HAI 1–3), and normal serum ALT for at least 12 mo, representing the final phase, the asymptomatic chronic carrier state. For comparison, sera from 23 HBsAg-negative healthy volunteers with normal liver function tests were tested as controls.

To analyze the time course of IL-12 and T helper cell responses in relation to HBeAg to anti-HBe seroconversion, 33 HBeAg positive, anti-HBe negative, and HBV DNA positive patients (15 from Group 1 and 18 from Group 2; male, 30; female, 3) were studied at regular intervals before, during, and after IFN- α treatment. The median follow up was 21 mo (range 10–48) and serial samples from individual patients were analyzed (median 13 samples per patient, range 8–20). These patients were part of a clinical trial carried out with Ethics Committee approval. Recombinant IFN- α 2b (Schering-Plough, Welwyn Garden City, UK) was given subcutaneously, 10 mega units, three times a week for 4 mo. A successful response to IFN- α was defined as a sustained loss of HBV DNA and HBeAg to anti-HBe seroconversion within 12 mo of completing treatment.

IL-12 and IL-12 p40 measurement. Serum levels of IL-12 heterodimer were determined by a specific ELISA using a rat monoclonal antibody (clone 20C2) reacting with a conformational epitope on the 75-kD heterodimer IL-12 molecule. The IL-12 p40 chain was determined using a different primary antibody (clone 4A1). Both assays were performed as previously described (12) with minor modifications. Briefly, 96-well microtiter plates (Nunc Maxisorp, Roskilde, Denmark) were coated with 100 μ l of the primary monoclonal antibody against IL-12 or IL-12 p40 (2.5 μ g/ml in 0.01 M bicarbonate

buffer, pH 9.6), and incubated overnight at 4°C. The coated plates were washed three times in deionized water and the remaining non-specific protein binding capacity of the wells was blocked with 0.5% bovine serum albumin in phosphate buffered saline, pH 7.4, overnight at 4°C. Serum samples, diluted 1:5, and serial dilutions of the recombinant standards (IL-12 or IL-12 p40) were added to the plates and incubated for 2 h at 20°C. After washing with PBS buffer containing 0.05% Tween 20, 100 μ l of secondary antibody (clone 4D6-POD, working concentration 125 ng/ml) was added for 2 h. The enzyme reaction was developed using 100 μ l of 10 mmol/liter tetramethylbenzidine, 80 mmol/liter H₂O₂, and 30 mmol/liter potassium citrate, pH 4.1. After a 10-min incubation at room temperature, the reaction was terminated with 50 μ l of 1 M H₃PO₄, and the optical density read at 450 nm (MR 700 automated plate reader; Dynatech Laboratories, Inc., Chantilly, VA).

Each sample was tested in duplicate and the values determined from the standard curve (range 0–800 pg/ml), constructed using the serial dilutions of recombinant IL-12 or IL-12 p40 standards. The detection limit was < 10 pg/ml with the intra- and inter-assay variances for both ELISAs being 6.3±2.5 and 11.3±3.5%, respectively.

The IL-12 heterodimer-specific ELISA showed no cross-reactivity with other recombinant cytokines or cytokine receptors (IL-12 p40, IL-2, IFN γ , IL-4, TNF- α , or recombinant TNF receptors) over a wide range of concentrations (0.1 pg/ml–1 μ g/ml). In patients with high serum levels of IL-12, ELISA testing was repeated with serial dilutions (1:5–625) to exclude the possibility of false positive results, which may arise with inadequate dilution. Furthermore, the specificity of the ELISA detection method was verified by the addition of recombinant IL-12 (50–500 pg/ml) to sera with high levels of IL-12. The detection of this added recombinant IL-12 confirmed that the high levels of endogenous IL-12 in certain samples was not due to interference with unknown serum components.

The IL-12 p40-specific ELISA showed 20–25% cross-reactivity with recombinant IL-12 heterodimer, demonstrating that the antibody epitope of the p40 chain was recognized as part of the IL-12 heterodimer. With the available antibody specific for IL-12 p40 (clone 4A1), it was not possible to differentiate various forms of IL-12 p40 (monomer, homodimer, or as part of the IL-12 heterodimer).

Assays for Th1 and Th2 cytokines. Serum IFN- γ , IL-2, and IL-4 levels were measured with sandwich ELISAs, each using two mAbs directed to different epitopes (for IFN- γ mAbs, clones 69 and 69 peroxidase-conjugated antibody (POD); for IL-2 mAbs, clones 3D5/7B1 and 13A6 POD; for IL-4 mAbs, clones 5 and 4F2 POD; kindly donated by Dr. H. Gallati, Hoffmann-La Roche, Basel, Switzerland) as previously described (2, 15). Serum samples, diluted 1:5, were compared with serial dilutions of recombinant standards IFN- γ (specific activity 2 × 10⁸ U/mg), IL-2, and IL-4. The incubation with the mAbs for IFN- γ and IL-2 was for 24 h at 2–8°C, and for IL-4 at 20–22°C. OD readings were performed at 450 nm. The ELISAs were sensitive to IFN- γ levels above 30 pg/ml, IL-2 levels above 20 pg/ml, and IL-4 levels above 15 pg/ml. The inter- and intraassay coefficients of variation

were 8 and 5% for the IFN- γ assay, 5–12 and 3–7% for the IL-12 assay, and 5–8 and 9% for the IL-4 assay. The specificity of the assays were confirmed by checking the assays against other human cytokines (500 pg/ml recombinant TNF- α , IL-1 α , IL-1 β , and IL-6), which showed no cross-reactivity.

PBMC proliferation assay. PBMCs were separated by standard density gradient centrifugation with 'Lymphoprep' (Nyegaard, Oslo, Norway). The cells were cultured at 2×10^5 cells/well in buffered RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated human AB serum (Lot 1652; Gemini Bio-Products Inc., Calabasas, CA) in 96-well, flat-bottomed sterile microtiter plates (Nunclon; GIBCO BRL, Glasgow, UK) for 5 d at 37°C in a humidified atmosphere of 5% CO₂ in air, as previously described (2). Briefly, the cell culture was performed in the presence or absence of 1 μ g/ml recombinant HBcAg (rHBcAg; Wellcome Research Laboratories, Beckenham, Kent, UK) in complete medium (buffered RPMI 1640 supplemented with 10% AB serum). Each well was pulsed with 0.5 μ Ci tritiated Thymidine (Amersham International, Little Chalfont, UK) 16 h before harvesting. The cells were washed and harvested onto glass fiber filters (Packard Instruments, Groningen, The Netherlands) using an automated cell harvester. The amount of radiolabel incorporated into DNA was measured by a direct beta counter (Matrix 9600; Packard Instruments). The proliferation assay was performed in 12 replicates and the results expressed as the mean counts per minute. The stimulation index (SI) was calculated by dividing the cpm for the antigen-stimulated cultures by that of control cultures (PBMCs with complete medium only). An SI greater than two times the mean plus 2 SD for the 23 healthy controls (i.e., an SI > 2.5) was considered significant.

To determine the HLA restriction of the PBMC proliferative response to recombinant HBcAg, blocking experiments with monoclonal anti-HLA antibodies (kindly provided by Dr. P. Banga, Department of Medicine, King's College, London) were performed. Monoclonal antibodies recognizing HLA class I (W6/32) and class II determinants to DR (L243), DP (B7/21), DQw1 (4D4), DQw2 (C1A2), and DQw3 (1VD12) were each added separately to the PBMC and antigen mixture, at three different final dilutions of 1:6, 1:12, and 1:24. The PBMC-proliferative response to rHBcAg was significantly inhibited in a dose-dependent manner by the antibody directed to HLA DR, demonstrating that the cellular response was HLA class II restricted.

Hepatitis serology. HBsAg, HBeAg, anti-HBe, anti-hepatitis Delta virus, anti-HCV, and anti-HIV were tested using commercially available assays (Abbott Diagnostics, Maidenhead, UK; Sorin Biomedica, Saluggia, VC, Italy, and UBI HCV EIA; United Biochemical Inc., Lake Success, NY). Serum HBV DNA was measured using a solution hybridization assay (Genostics; Abbott Diagnostics, Maidenhead, UK).

Statistical analysis. Nonparametric tests were used to analyze the results of the SI and the serum levels of IL-12, IL-12 p40, IFN- γ , IL-2, and IL-4. In particular, the Mann-Whitney U test was employed to compare data between two groups and the nonparametric Wilcoxon's matched pairs test was used to compare two sets of data in the same group. Correlation analysis was performed using the Spearman's rank-regression coefficient. The Chi-square test (χ^2) was used to compare the prevalence or distribution of two variables in the same group. Data are presented as mean \pm SEM. $P < 0.05$ was considered statistically significant.

Results

Serum levels of IL-12, IL-12 p40, and Th cytokines. The serum levels of both IL-12 and IL-12 p40 subunit were significantly higher in the cohort of patients with chronic HBV infection studied ($n = 72$) in comparison with control subjects (mean \pm SEM for IL-12, 57.5 \pm 8.8 vs. 9.9 \pm 1.1 pg/ml; IL-12p40, 147.1 \pm 10.2 vs. 78.8 \pm 6.1 pg/ml, $P < 0.01$ Mann Whitney-U). Normally, the production of IL-12 p40 heavy chain exceeds the

production of IL-12 heterodimer between 10- and 50-fold (6). To evaluate the proportion between IL-12 p40 and the biologically active IL-12 heterodimer, we calculated the ratio between their values detected at the same time point in individual patients and controls. Patients with chronic HBV infection showed a significantly lower ratio of IL-12 p40/IL-12 in comparison with noninfected controls (6.1 \pm 0.8 vs. 12.1 \pm 1.6, mean \pm SEM, $P < 0.01$ Mann Whitney-U). When this ratio was analyzed between different groups of chronic HBV carriers, there was no significant difference between the three groups of patients irrespective of their histological activity or virus replication (IL-12 p40/IL-12 heterodimer ratio for groups 1, 2, and 3, 5.5 \pm 0.9, 4.4 \pm 0.8, and 7.1 \pm 1.9, respectively, mean \pm SEM).

The serum levels of the Th1 cytokines IFN- γ and IL-2 were significantly higher in patients with chronic hepatitis B infection compared with controls (mean \pm SEM for IFN- γ , 75.1 \pm 8.6 vs. 5.5 \pm 0.7 pg/ml; for IL-2, 3.7 \pm 0.4 vs. 1.2 \pm 0.1 ng/ml, respectively, $P < 0.01$ Mann Whitney-U). The analysis between different groups of chronic HBV carriers showed that patients with high necroinflammatory activity (group 2) have significantly higher serum levels of both IL-2 and IFN- γ when compared with chronic HBV carriers with minimal histological activity (groups 1 and 3) or to control subjects (for all $P < 0.01$, Fig. 1). The serum levels of both IL-2 and IFN- γ in patients from group 1 were higher than controls ($P < 0.01$), while in patients from group 3, only IFN- γ levels differed significantly from the levels detected in controls. There was no difference in the serum levels of IL-4 between chronic HBV carriers and controls (92.7 \pm 14.1 and 74.8 \pm 2.8 pg/ml, respectively, mean \pm SEM).

Longitudinal analysis of IL-12 and IL-12 p40 in chronic HBV infection. 10 of 33 patients responded to IFN- α with HBeAg to anti-HBe seroconversion and sustained loss of serum HBV DNA within 12 mo of completing treatment (three from group 1 and seven from group 2). 8 of these 10 patients

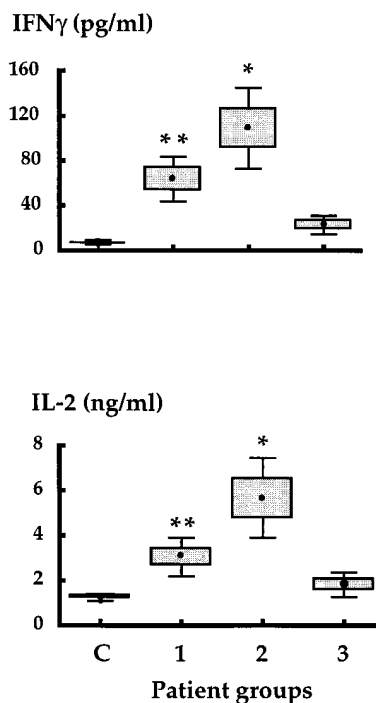


Figure 1. Serum levels of Th1 cytokines in three groups of chronic HBV carriers. C, control subjects; group 1, patients with active viral replication and minimal histological activity; group 2, patients with active viral replication and moderate to severe necroinflammatory activity; group 3, HBsAg-positive patients without viral replication and no necroinflammatory activity. *Significantly higher than groups 1 and 3, and controls ($P < 0.01$); **Significantly higher than group 3 and controls.

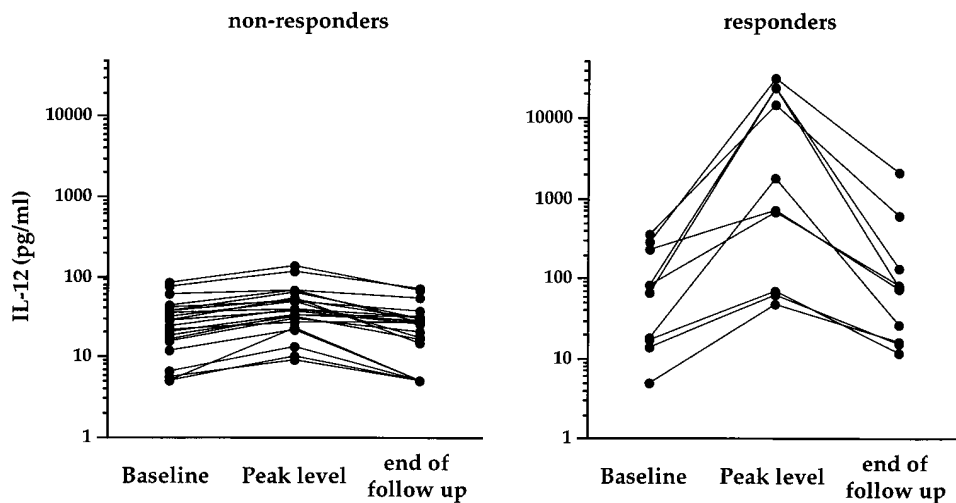


Figure 2. Serial analysis of serum levels of IL-12 in chronic HBV carriers in relation to response to IFN- α treatment. The serum levels of IL-12 heterodimer are shown before the treatment (*Baseline*), the peak serum IL-12 detected during the period of the study and 12 mo after the end of treatment (*end of follow up*). IL-12 levels increased more than 2.5-fold in all patients with chronic hepatitis B who seroconverted to anti-HBe, while this was observed in only one of the 23 nonresponders.

developed a characteristic hepatitis flare in association with IFN- α treatment (peak ALT level, 396.5 ± 80.3 IU/ml, mean \pm SEM), which preceded the time of seroconversion. A similar flare was observed in 15 of the 23 nonresponders to IFN- α therapy (peak ALT level, 283.1 ± 59.8 IU/ml, mean \pm SEM).

The pretreatment serum levels of the IL-12 heterodimer were significantly higher among the responders to IFN- α in comparison with the nonresponders (pretreatment IL-12, 116.9 ± 39.6 vs. 28.1 ± 4.5 pg/ml, respectively, $P < 0.01$, Mann-Whitney U test), whereas there was no significant difference between the pretreatment IL-12 p40 levels. The pretreatment IL-12 p40/IL-12 heterodimer ratio was significantly lower among the responders compared with nonresponders (2.8 ± 0.8 vs. 8.1 ± 1.4 , mean \pm SEM) ($P < 0.01$, Mann-Whitney U test). Furthermore, all 10 patients who seroconverted to anti-HBe showed > 2.5 -fold increase in serum IL-2 either during treatment or in the posttreatment follow-up period. In 7 of these 10 patients, the peak serum IL-12 reached levels > 500 pg/ml (Fig. 2). Among the 23 nonresponders, only one patient developed a rise in serum IL-12 > 2.5 -fold above the corresponding pretreatment level (from 5 pg/ml at baseline to 20 pg/ml peak level) and in none of the nonresponders did the peak serum

IL-12 level reach above 200 pg/ml (Chi-square 20.4, $P < 0.01$). The peak IL-12 level in the 10 responders was significantly higher in comparison with the peak level reached by the 15 nonresponders who had an ALT flare ($12,232.7 \pm 4,635.3$ vs. 50.9 ± 9.4 pg/ml, mean \pm SEM, $P < 0.01$, Mann-Whitney U test).

Although the IL-12 p40 levels showed some increase during the period of longitudinal analysis, there were no significant differences between the changes observed in the responders and nonresponders (Fig. 3). The IL-12 p40/IL-12 heterodimer ratio was substantially reduced at the time of the peak serum IL-12 concentration among the 10 patients who seroconverted to anti-HBe as there was no parallel increase in IL-12 p40. In contrast, the ratio remained unchanged in the nonresponders, irrespective of whether or not there was an ALT flare (Fig. 4).

Patients who seroconverted to anti-HBe showed a marked elevation in the serum levels of IL-2 and IFN- γ either during ($n = 7$) or after ($n = 3$) the end of treatment. In 7 of the 10 responders, the increase of serum IFN- γ level was $> 2.5\times$ in comparison with the corresponding pretreatment level, whereas only 3 of the 23 nonresponders had such a significant increase in serum IFN- γ (Chi-square 10.7, $P < 0.01$) (Fig. 5). Similarly,

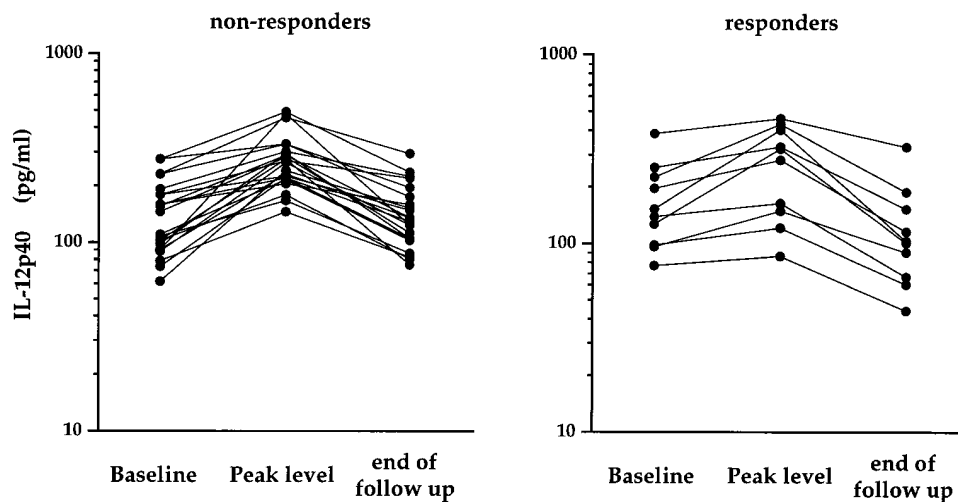


Figure 3. Serial analysis of serum levels of IL-12 p40 in chronic HBV carriers in relation to the response to IFN- α treatment. There were no significant differences between patients who seroconverted to anti-HBe (*responders*) and nonresponders to IFN- α treatment.

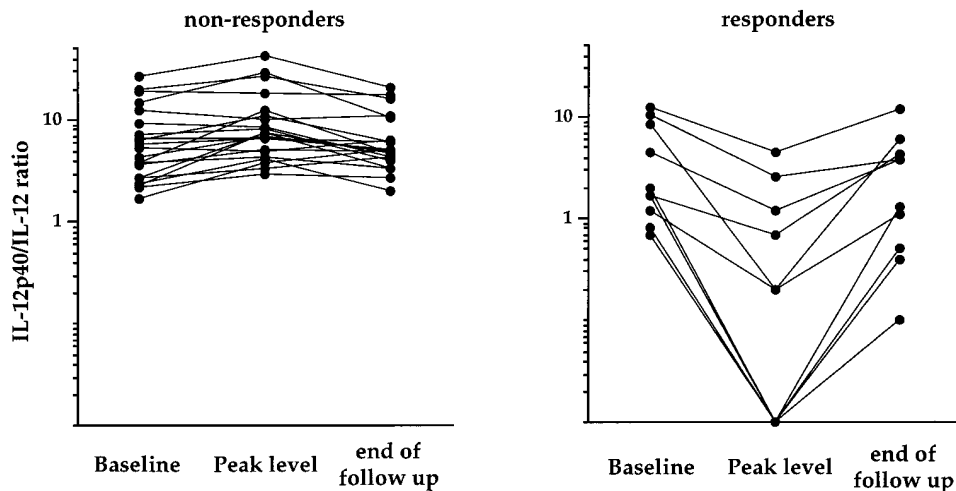


Figure 4. Changes in the ratio of serum IL-12 p40/IL-12 heterodimer during serial analysis of chronic HBV carriers treated with IFN- α . At the time of the highest serum IL-12 level for each patient, the ratio decreased significantly ($P < 0.01$) only in patients who seroconverted to anti-HBe.

a significant rise in serum IL-2 levels ($> 2.5\times$ pretreatment level) was observed in 7 of 10 responders during or immediately after IFN- α treatment. In 8 of 10 patients, the peak serum IL-2 level was > 5 ng/ml. In contrast, only 5 of the 23 nonresponders had peak serum IL-2 levels above 5 ng/ml (Chi-square 9.9, $P < 0.01$) (Fig. 6).

HBcAg-specific proliferative response during IFN- α treatment. 23 patients developed a significant rise in HBcAg-specific T cell-proliferative response during IFN- α treatment (8 responders and 15 nonresponders), and all of them showed a hepatitis flare. There was no difference in the peak SI between responders ($n = 8$) and nonresponders ($n = 15$) (peak SI, 4.87 ± 0.46 and 4.17 ± 0.49 , respectively, mean \pm SEM). In contrast, only 2 of the remaining 10 patients who did not show an ALT flare developed an increase in T cell-proliferative response during IFN- α treatment (peak SI, 2.15 ± 0.2 , mean \pm SEM, $n = 10$). Among the 23 patients with a significant rise in the HBcAg-specific proliferative response, the peak SI preceded the peak of the ALT rise in 16 patients, with a mean interval between the peak SI and peak ALT of 6.9 ± 1.1 wk. In

the remaining seven patients, the peak SI coincided with the peak of serum ALT.

Time course of cytokine profiles in relation to hepatitis flare and viral clearance. In the eight patients who developed an ALT flare and seroconverted to anti-HBe, we analyzed the sequence of events in relation to serum levels of the cytokines. In all eight patients, there was a significant rise in serum IL-12 with the peak IL-12 level following the ALT peak by a mean period of 9.8 ± 2.8 wk. When this IL-12 rise was analyzed in relation to the time of HBeAg to anti-HBeAg seroconversion, the serum IL-12 peak occurred either simultaneously ($n = 2$) or preceded the seroconversion by 9.3 ± 1.0 wk ($n = 6$). In all eight patients, the IL-12 levels declined rapidly thereafter. At the time of serum IL-12 rise, there was a significant increase in serum IFN- γ and IL-2 levels that also preceded or coincided with the time seroconversion (Fig. 7). In patients who did not seroconvert, no significant changes in the serum levels of IL-12, IFN- γ , or IL-2 were detected during the follow-up period, regardless of whether there was or wasn't an ALT flare (Figs. 8 and 9).

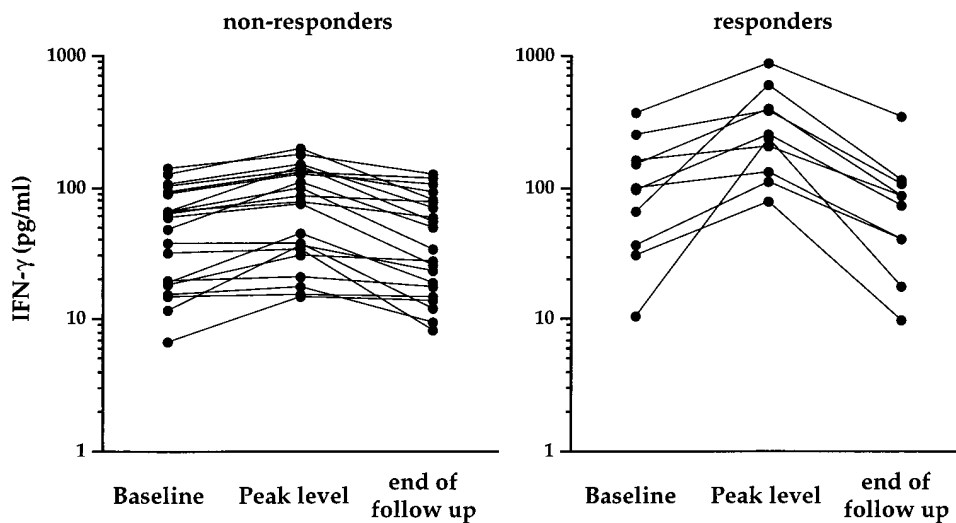


Figure 5. Longitudinal analysis of IFN- γ serum levels in chronic HBV carriers in relation to the outcome of IFN- α treatment. The majority of patients who seroconverted to anti-HBe showed > 2.5 -fold increase in IFN- γ level in contrast with nonresponders.

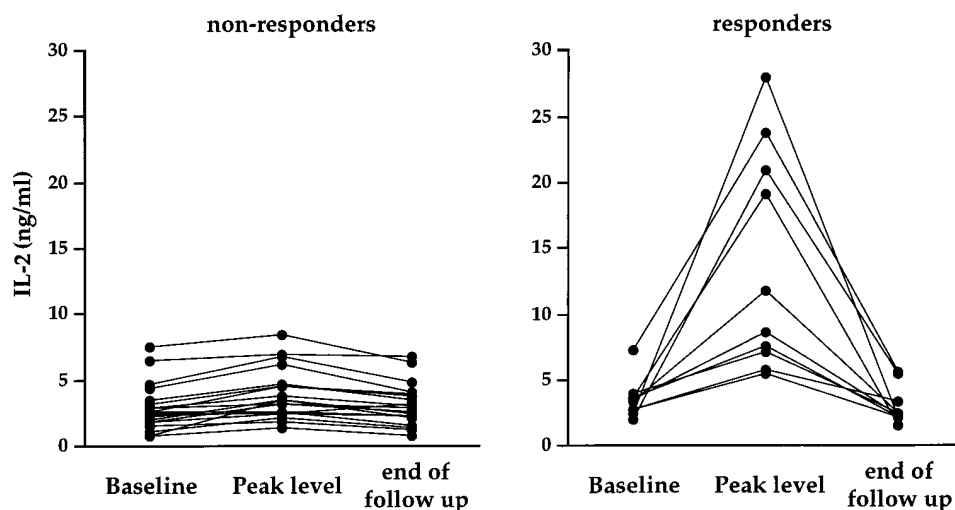


Figure 6. Longitudinal analysis of serum IL-2 levels in chronic HBV carriers in relation to the outcome of IFN- α treatment. Serum IL-2 levels increased significantly in patients with chronic hepatitis B who seroconverted to anti-HBe.

Discussion

The present study indicates that, although immune-mediated hepatocytolysis is a critical initial event in the process of viral clearance, a substantial increase in IL-12 production, along with the induction of Th1 cytokines (IFN- γ , IL-2), is required for a sustained immune control over HBV replication, manifested by seroconversion to anti-HBe. Thus, these results provide the first in vivo evidence that IL-12 may contribute to viral clearance in chronic HBV infection.

The finding that the peak of serum IL-12 levels in patients who seroconverted to anti-HBe occurred consistently after the hepatitis flare was unexpected. Several elements in the longitudinal analysis demonstrate the specificity of this observation. First, the substantial increase in IL-12 production practically occurred only in patients who seroconverted to anti-HBe and during the long follow-up period (median 21 mo, range 10–48) this was the only peak in serum IL-12 in each of these patients. The IL-12 rise was associated with a significant increase in Th1 cytokines, and high levels of IFN- γ and IL-2 were observed in these patients. Second, despite the fact that a similar degree of hepatocytolysis developed in 15 of the other 23 interferon-treated patients who remained seropositive for HBeAg, only one showed some IL-12 elevation during the entire follow-up. Third, the specificity of ELISA detection of IL-12 heterodimer was carefully verified by the lack of cross-reactivity with other cytokines, testing serial dilutions of the positive samples and by spiking experiments with the addition of recombinant IL-12.

The dissociation between the mechanisms responsible for the immune-mediated hepatocytolysis and for viral clearance in HBV infection was first proposed by F. Chisari and his group, based on their work with a transgenic mouse model (16). It was demonstrated that the interaction between antigen-specific CTL and target hepatocytes results in spotty necrosis, which is limited to a very few hepatocytes (17). Instead, the antiviral effect is mediated by IFN- γ , IL-2, and TNF- α released by HBV-specific CTLs, and these cytokines profoundly suppress HBV gene expression in infected hepatocytes by noncytolytic mechanisms (18–21). In a recent study using transgenic mice that replicate the entire HBV genome, they have further demonstrated that IFN- γ and TNF- α , secreted by

CTL or by antigen nonspecific macrophages, activate two independent virocidal pathways; the first eliminates HBV nucleocapsid particles and the second destabilizes the viral RNA (22). Thus, after recognition of HBV antigens on the surface of infected hepatocytes, CTLs perform two distinct functions; they kill a small fraction of infected hepatocytes and they secrete IFN- γ and TNF- α , which exert antiviral effects without destruction of hepatocytes. Effective clearance of duck HBV and woodchuck hepatitis virus has also been shown to occur without massive hepatocellular necrosis (23, 24).

The results in the present study demonstrate that a dissociation between immune-mediated hepatocytolysis and viral clearance also exists in patients with chronic HBV infection. Although we have not studied the local cytokine production in HBV-infected livers, the advantage of our longitudinal analysis is that it provides an understanding of the temporal relationship between changes in HLA class II-restricted T cell responses, the levels of heterodimer IL-12, IL-12 p40, and Th1 cytokines in relation to hepatocellular necrosis and HBV clearance in patients with chronic HBV infection. Both spontaneous or interferon-induced exacerbations of hepatocellular necrosis in chronic hepatitis B have previously been shown to be associated with an induction of HBeAg-specific, CD4+ T cell response (2, 3, 25). In agreement with these observations, the virus-specific T helper cell proliferation was enhanced in all 23 patients with hepatitis flare in the present study, irrespective of whether or not HBe seroconversion followed, and occurred either before or simultaneously with the ALT peak. In contrast, a significant increase in IL-12 production was observed only in patients who cleared the virus. This rise in serum IL-12 and Th1 cytokines always followed the ALT flare, but preceded the time of HBeAg to anti-HBe seroconversion. IL-12 is produced by phagocytic cells, B lymphocytes, and other antigen-presenting cells (6). A possible explanation of why the IL-12 peak occurs after the ALT flare would be that hepatocellular necrosis, induced by CTL, leads to the recruitment of macrophages and noncommitted T helper cells in the liver. Native particles of HBeAg are released from damaged hepatocytes and provide potent antigenic stimulation for these cells. In patients able to respond with an increase in IL-12 production, this will promote Th1 cell development and will

Responder with ALT flare

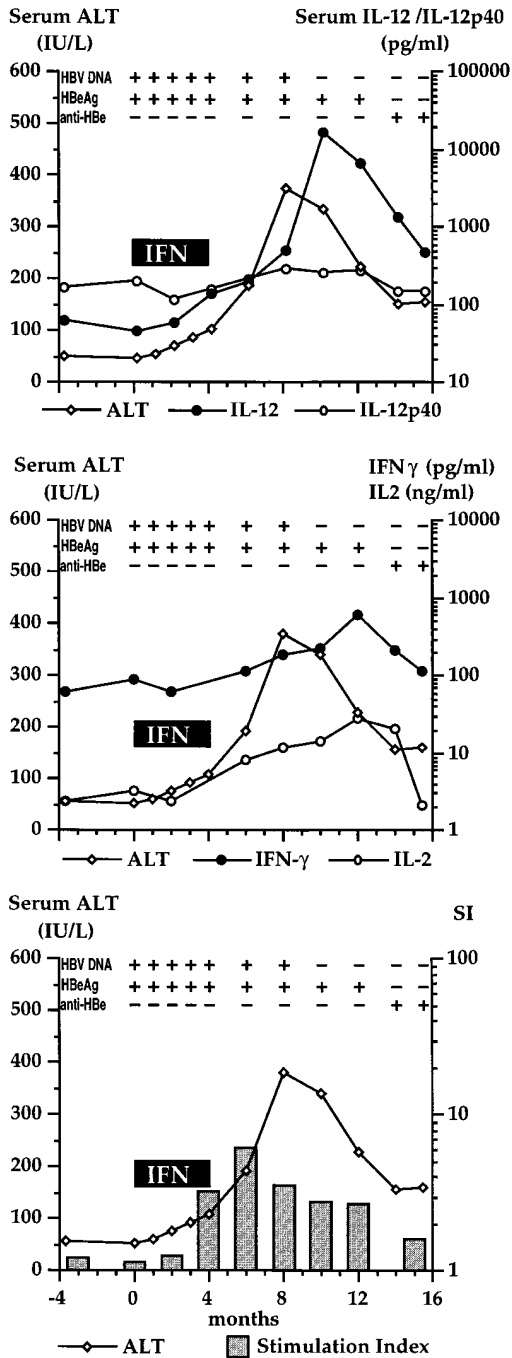


Figure 7. Time-course analysis of IL-12, IL-12 p40, Th1 cytokines, and HBcAg-specific T cell-proliferative response in relation to hepatocytolysis in a chronic HBV carrier with HBeAg to anti-HBe seroconversion as a result of IFN- α treatment (IFN, treatment with IFN- α). The peak of serum IL-12 was associated with a significant rise in serum IL-2 and IFN- γ level. The IL-12 peak preceded the time of seroconversion to anti-HBe.

stimulate the production of IFN- γ and TNF- α , which will exert their noncytolytic antiviral effects. Importantly, responders to IFN- α therapy had significantly higher pretreatment levels of serum IL-12 in comparison with chronic HBV carriers who

Non-Responder with ALT flare

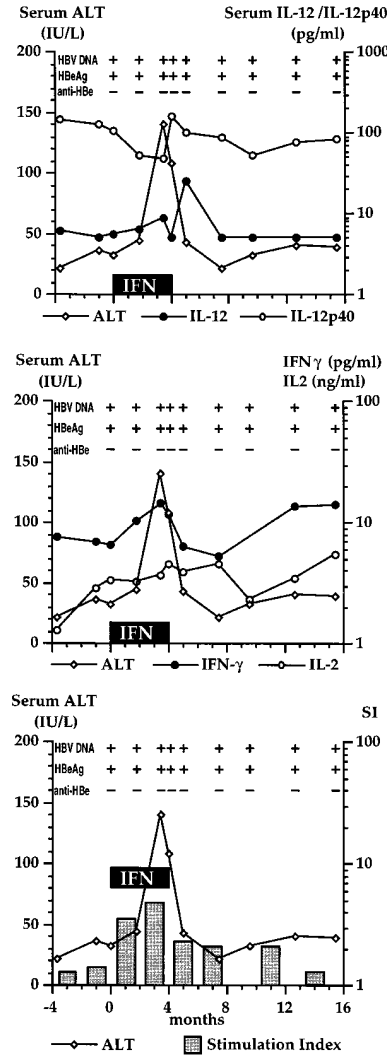


Figure 8. Time-course analysis of IL-12, IL-12 p40, Th1 cytokines, and HBcAg-specific T cell-proliferative response in a chronic HBV carrier with significant hepatocytolysis during IFN- α treatment but without seroconversion to anti-HBe.

failed the treatment. Further investigations are needed to determine the factors responsible whether or not a chronic HBV carrier will augment the IL-12 production sufficiently to induce viral clearance as a result of IFN- α treatment. Another marker of a direct stimulation of B cells by HBcAg released from damaged hepatocytes is the rise of serum IgM anti-HBc during exacerbations of chronic hepatitis B, and we have previously shown that the peak IgM anti-HBc follows the peak of hepatocytolysis by 3–8 wk (26).

The bioactive molecule of IL-12 is a heterodimer, produced along with its larger component, IL-12 p40, which is highly regulated and secreted in response to bacteria, bacterial products, and intracellular pathogens (6). The present study is the first report on serum IL-12 and IL-12 p40 levels and the kinetics of their changes during the course of chronic HBV infection. The longitudinal analysis revealed that before HBe seroconversion there is a rapid and substantial increase in the IL-12 heterodimer in the serum, which for a short period of time results in an inverse IL-12 p40/IL-12 ratio. All chronic HBV carriers have very high serum levels of anti-HBc antibodies and detectable levels of anti-HBe and -HBs were found in > 50% of

Non-Responder without ALT flare

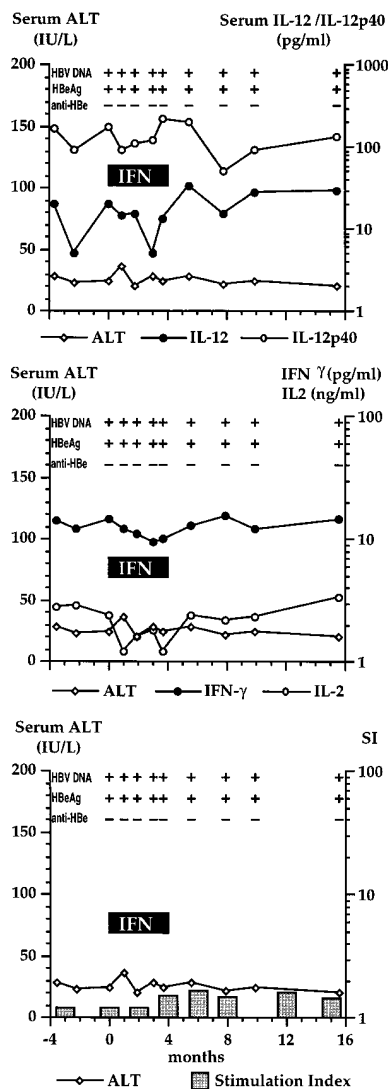


Figure 9. Time-course analysis of IL-12, IL-12 p40, Th1 cytokines, and HBcAg-specific T cell-proliferative response in a chronic HBV carrier with no ALT flare and no response to IFN- α treatment.

the asymptomatic viremic patients, indicating a Th2 predominance in chronic HBV infection (8, 27). A switch to the Th1 type of response would therefore be beneficial and would promote the cellular immune mechanisms essential for viral clearance. In transgenic mice expressing HBeAg, it has been shown that systemic application of recombinant IL-12 results in a shift of Th2-mediated responses towards predominance of Th1 cytokine profile (28). Our in vivo data indicate that IL-12 production is not impaired in patients with chronic HBV infection, but a substantial rise in IL-12 production was necessary to induce Th1 cytokine response and ultimately HBe seroconversion. The induction of endogenous IL-12 production was not a direct consequence of the IFN- α treatment, as it occurred only in patients with HBe seroconversion, but not in interferon nonresponders. In vitro data also demonstrate that IFN- α does not stimulate directly IL-12 priming for Th1 development (29). The observed strong association between the increase in serum levels of IL-12 and HBe seroconversion provides a rationale for the therapeutic use of IL-12 in patients with chronic hepatitis B. Thus, the present findings in patients

with chronic HBV infection support a previously proposed combination strategy with IL-12 plus therapeutic vaccination (28). Based on the ability of IL-12 to induce antigen-dependent Th1 differentiation and CTL proliferation (30), a combination of recombinant IL-12 and recombinant, particulate HBeAg may provide a specifically targeted immunotherapy in chronic carriers with ongoing HBV replication.

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