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CLINICAL RESEARCH

Ribavirin and IFN-α combination therapy induces CD4+ T-cell proliferation and Th1 cytokine secretion in patients with chronic hepatitis B

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Abstract

AIM: To investigate the anti-viral mechanism of combination therapy of interferon (IFN)-α and ribavirin in patients with chronic hepatitis B.

METHODS: Twenty patients were assigned to receive either IFN-α plus ribavirin (group A, *n* = 14) or no treatment as a control (group B, *n* = 6). Patients were analyzed for T-cell proliferative responses specific for hepatitis B virus (HBV)-antigen and cytokine production by peripheral blood mononuclear cells (PBMCs).

RESULTS: Combination therapy induced HBV-antigen specific CD4+ T-cell proliferative responses in four patients (28.6%). Production of high levels of HBV-specific IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-12 by PBMCs was found in five patients (35.7%), who showed significantly lower HBV DNA levels in serum at 12 mo after treatment ended (P = 0.038) and at 24 mo of follow-up (P = 0.004) than those without high levels of cytokine production.

CONCLUSION: HBV-antigen specific CD4+ T cells may directly control HBV replication and secretion of anti-viral T helper 1 (Th1) cytokines by PBMCs during combination therapy of chronic hepatitis B with ribavirin and IFN-α.

Key words: Hepatitis B; Interferon-alpha; Ribavirin; CD4+ T cells; Th1

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INTRODUCTION

More than 400 million people worldwide have chronic hepatitis B virus (HBV) infections[1]. Chronically infected patients with active liver disease have a high risk of developing cirrhosis and hepatocellular carcinoma[2]. However, therapeutic options against HBV still present a major clinical challenge. The goal of treatment is HBV DNA suppression, normalization of alanine aminotransferase (ALT) levels and reduction in liver necroinflammation. Currently available therapies against HBV are mainly interferon (IFN)-α and nucleoside analogs, which are well tolerated and induce a decrease in serum HBV DNA levels and normalization of serum ALT levels. However, the efficacy of IFN-α[3,4] or nucleoside analogs for treatment of hepatitis B varies in different clinical situations[5-12]. IFN-α shows seroconversion from hepatitis B e antigen (HBeAg) to antibody to HBeAg (anti-HBe), concomitant with HBV DNA negativity in just one-third of patients treated, and is both costly and induces adverse effects[3]. It has been well established that IFN-α has potent antiviral activity against DNA and RNA viruses, and that it also acts as an immunomodulatory agent[13]. Some reports have suggested that ribavirin shows antiviral and immune effects against various infections[14], including hepatitis B and C. Both drugs have the capacity to modulate systemic as well as virus-specific T-cell responses, along with the potential to shift the profile of cytokine secretion[15,16].

Recent reports have suggested that combination therapy with IFN-α plus ribavirin for chronic hepatitis B significantly reduces viremia[17,18] and induces lasting CD4+ T-cell proliferation and Th1 cytokine release at the site of infection, which may lead to sustained HBV eradication[18]. These preliminary data in anti-HBe-positive patients refractory to IFN-α treatment appear to be promising[18]. Thus, in the present study, we investigated the mechanism involved in the control of HBV replication, utilizing combination therapy with ribavirin and IFN-α.

MATERIALS AND METHODS

Patients

Twenty patients with chronic hepatitis B (14 men and 6 women; mean age 42 years), positive for both anti-HBe and HBV DNA in the serum, and who had failed previous IFN-α treatment were enrolled in this prospective trial. None had human immunodeficiency virus, hepatitis

C virus, or hepatitis D virus infections, hepatocellular carcinoma, or had received nucleoside analogs. Six healthy controls were also analyzed (mean age 38 years). Table 1 shows patient characteristics at enrollment. This study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by institutional ethics committee. Informed consent was obtained from all patients prior to inclusion.

Therapeutic and analytic schedule

The patients were divided into two groups: group A (n = 14) for combination therapy with IFN-α plus ribavirin, and group B (n = 6) for untreated controls. Patients in group A received 5 million U IFN-α2b three times a week for 12 mo, plus ribavirin (1000 mg/d) orally for 12 mo[17]. Patients were followed for 12 mo after the end of treatment. Blood chemistry, blood cell counts, and HBV DNA were measured at the beginning of treatment, and then every 1-2 mo during treatment and follow-up periods. HBeAg and anti-HBe were measured at the beginning of treatment, and then at 6-mo intervals. Blood samples for immunological analysis were collected before therapy and at 3, 6, 9, 12, 18 and 24 mo after the commencement of therapy. Serum HBV DNA was quantified, using transcription-mediated amplification and a hybridization assay. The concentration of HBV DNA in the samples was expressed as the logarithm of genome equivalents per milliliter (LGE/mL). Biochemical and hematologic parameters were measured by standard methods. All patients completed treatment and follow-up.

Cell preparation

The methods of cell preparation used here were nearly identical to those of Ren *et al*[19] in their report focusing on therapeutic vaccination against chronic hepatitis B. Peripheral blood mononuclear cells (PBMCs) from 20 patients were separated from heparinized blood by density-gradient centrifugation with lymphoprep (Nycomed Pharma AS, Oslo, Norway). B cells were removed from PBMCs by negative depletion, by incubating the cells with mouse anti-$CD19^+$ antibodies coated on magnetic beads (Danal, Olso, Norway). CD4+ or CD8+ T cells were then removed from the resultant T cells in the same manner, using mouse anti-human CD4+ or CD8+ antibodies coated on magnetic beads (Danal), respectively. CD4+ cells were also blocked with CD4+ antibodies (Caltag Laboratories, Burlingame, CA, USA). The purity of the T-cell subpopulation was monitored by immunolabeling with anti-$CD3^+$ antibodies (Becton Dickinson, San Jose, CA, USA). Flow cytometry revealed a > 95% purified T-cell subpopulation. PBMC or lymphocyte subsets were resuspended with 2 mmol/L l-glutamine, 10 mmol/L HEPES, 100 kU/L penicillin, 100 mg/L streptomycin, and 50 mL/L human AB serum (complete medium).

Proliferation assay

The proliferation assay used in this study was nearly identical to that of Ren *et al*[19]. T cells (1.5×10^6 cell/L in 0.2 mL complete medium) were cultured in triplicate wells of 96-well round-bottom microplates with medium alone, were stimulated with 10 mg/L phytohemagglutinin (PHA: Sigma) and 3 mg/L HBV antigens: HBsAg protein, synthetic entire preS1, HBeAg, and hepatitis B core antigen (HbcAg) (Virostat, Portland, ME, USA). After 4 d of culture at 37℃ in an atmosphere of 50 mL/L CO_2 in air, the cells were labeled for 18 h with 37 kBq of [^{3}H]-thymidine (Amersham, Little Chalfont, UK). DNA-incorporated radioactivity was measured by scintillation counting. Data were expressed as the stimulation index (SI), calculated as the ratio of the mean cpm of triplicate cultures obtained in the presence of antigen to cpm obtained without antigen. SI > 3 was considered significant. The proliferative responses were not tested with PBMCs from patients in control group B.

Table 1 Clinical characteristics of patients at enrollment

Patient No.	Age (yr)	Gender	ALT (nkat/L)	HBV DNA (10^3 LGE/L)	Type of response (end of treatment)
Group A					
1	48	M	89	6.8	Responder
2	51	M	158	7.3	Responder
3	44	M	86	8.3	Responder
4	41	M	464	8.0	Responder
5	39	M	122	4.8	Responder
6	67	M	142	8.1	Non-responder
7	52	M	140	8.3	Non-responder
8	26	M	69	4.8	Non-responder
9	52	M	46	7.6	Non-responder
10	33	M	39	5.4	Non-responder
11	24	F	458	8.7	Non-responder
12	27	F	51	8.5	Non-responder
13	52	F	80	7.4	Non-responder
14	37	F	57	6.8	Non-responder
Group B					
15	42	F	43	7.2	No treatment
16	33	F	19	8.1	No treatment
17	51	M	138	3.9	No treatment
18	31	M	57	7.1	No treatment
19	29	M	79	7.1	No treatment
20	44	M	46	7.2	No treatment

P > 0.05, Group A *vs* Group B.

Cytokine assay

The cytokine assay used in this study was nearly identical to that of Ren *et al*[19]. PBMCs (1.5×10^6 cell/L in 0.2 mL complete medium) were cultured in triplicate wells of 96-well round-bottom microplates with medium alone, and stimulated with 10 mg/L PHA or with 3 mg/L HBsAg, HBeAg, HBcAg or preS1. After 3 d of culture at 37℃ in an atmosphere of 50 mL/L CO_2 in air, culture supernatants were collected. Concentrations of IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-10 and IL-12 p70 were determined using commercial ELISA kits (Genzyme, Cambridge, MA, USA). Production levels after antigen stimulation were expressed as the ratio of the mean cytokine concentration of triplicate cultures obtained in the presence of antigen to that obtained without antigen.

Statistical analysis

Results are expressed as mean ± SD. Differences in proportions were tested by the χ^2 test. Mean quantitative values were compared using the Mann-Whitney U test. All reported P values were two-tailed and $P \leqslant 0.05$ was considered significant.

Table 2 SI of T cells against HBV antigens from combination therapy patients

Patient No.	12 mo					24 mo				
	PHA	HBsAg	preS1	HBeAg	HBcAg	PHA	HBsAg	preS1	HBeAg	HBcAg
1	38.1[1]	3.5[1]	3.2[1]	3.9[1]	4.2[1]	8.2[1]	4.1[1]	3.5[1]	4.1[1]	4.8[1]
2	20.3[1]	3.4[1]	4.0[1]	4.1[1]	4.4[1]	7.7[1]	3.2[1]	3.0	4.4[1]	4.7[1]
3	19.5[1]	3.2[1]	3.9[1]	4.8[1]	4.1[1]	4.9[1]	3.5[1]	3.7[1]	4.7[1]	5.1[1]
4	32.3[1]	4.3[1]	7.8[1]	7.2[1]	7.9[1]	9.6[1]	4.0[1]	6.9[1]	8.1[1]	8.8[1]
5	15.5[1]	1.9	2.3	2.0	1.7	8.4[1]	3.5[1]	3.5[1]	4.3[1]	4.4[1]
6	12.8[1]	0.4	1.2	1.5	1.4	4.9[1]	1.1	1.3	1.5	1.2
7	50.2[1]	2.1	1.9	2.0	2.0	38.8[1]	1.7	1.8	1.6	1.6
8	4.9[1]	1.8	2.1	1.7	1.9	NT	NT	NT	NT	NT
9	66.3[1]	2.0	1.5	1.8	1.6	12.0[1]	1.5	1.2	1.5	1.3
10	56.6[1]	2.3	2.3	2.0	2.1	18.3[1]	2.0	2.1	1.8	1.9
11	23.8[1]	1.1	1.0	1.1	1.2	8.7[1]	1.0	0.8	1.2	1.3
12	19.2[1]	1.4	1.2	1.4	1.3	4.6[1]	1.1	1.5	1.1	1.2
13	42.0[1]	1.8	1.5	1.9	1.7	6.9[1]	1.5	1.3	1.4	1.5
14	21.8[1]	2.2	2.4	2.2	2.6	4.9[1]	2.0	2.3	1.9	2.0

[1]SI > 3 correspond to significant proliferative responses. NT, not tested; PHA (10 mg/L); HBV antigen (3 mg/L).

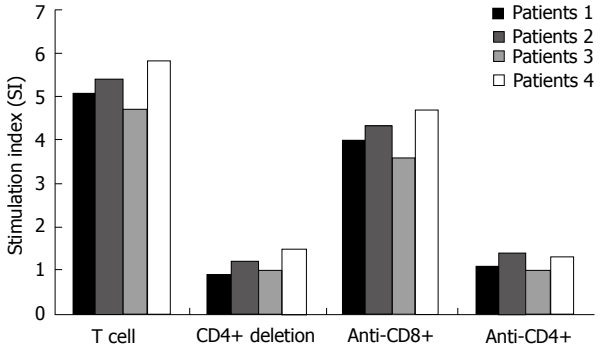

Figure 1 Abrogation of antigen-specific T-cell proliferative responses from patients 1-4 at 12 mo ([b]$P < 0.01$ *vs* T cell and Anti CD8+).

RESULTS

Clinical outcome

HBV DNA levels decreased, with a significant reduction at 9 mo and thereafter, as compared to those at baseline, in the combination therapy patients. Serum HBV DNA levels were also significantly lower in the combination therapy patients than in the controls at 12 and 24 mo. At 12 mo, four patients in group A (28.6%) (patients 1-4) had undetectable HBV DNA levels, and also showed sustained normalization of ALT; they were thus considered sustained responders, as previously reported[17,18]. The remaining 10 patients at 12 mo had detectable HBV DNA and elevated ALT levels.

Induction of T-cell proliferation response to HBV antigens

Proliferative responses of T cells during combination therapy and the follow-up period are summarized in Table 2. Four patients (1-4) showed significant proliferative responses at 12 mo, and these responses were sustained until 24 mo (the end of follow-up). These proliferative responses were always specific to both HBV antigens. Thus, this combination therapy was found to have induced proliferative T cell responses specific to the antigen contained in these four patients (28.6%). Patient 5 also showed a strong proliferative response at 24 mo. However, whether the combination therapy induced this response is unclear because it occurred at 12 mo after completion of therapy.

T-cell proliferative responses were also examined for patients 1-4 after incubation with anti-CD4+ antibodies or removing CD4+ or CD8+ cells. Depletion of CD8+ cells did not clearly inhibit the proliferative responses, while depletion of CD4+ cells or blocking with anti-CD4+ antibodies completely abrogated the proliferative responses of the patients (Figure 1).

HBV-specific cytokine production in PBMCs

HBV-specific cytokine production levels of PBMCs are shown in Figure 2. Cytokine production showed a Th1-like pattern characterized by secretion of IFN-γ, TNF-α, and IL-12 in the absence of IL-4 and IL-10 in five patients (1-5, defined as responders). Patients 1-4 exhibited remarkable increases in IFN-α, TNF-α, and IL-12 production at 3, 6 or 9 mo, as compared to patient 5 who showed mild, but not significant, proliferative responses (Table 2). These responses were sustained until the end of the observation period. Production of Th1 (IFN-γ, TNF-α, and IL-12) and Th2 (IL-4 and IL-10) cytokines did not increase, or remained unchanged in the patients who received other combination therapy (patients 6-14, defined as non-responders).

The mean serum HBV DNA level was lower in responders than in non-responders at 12 mo (4.3 ± 1.1 *vs* 6.0 ± 1.1, $P = 0.038$) and 24 mo (3.8 ± 0 *vs* 5.7 ± 1.2, $P = 0.004$). It is noteworthy that HBV DNA fell to under the detection limit at 24 mo in all responders. The decrease in serum HBV DNA level was almost coincident with the increase in IFN-γ production by HBV antigen-specific T cells, but was not preceded by any increase in serum ALT levels in four responders (patients 2-5).

DISCUSSION

In the present study, a significant decrease was found in serum HBV DNA levels at 9 mo and thereafter, along with significantly lower levels of HBV DNA in the combination therapy patients than in the controls at 12 and 24 mo. IFN-α plus ribavirin therapy appeared to inhibit

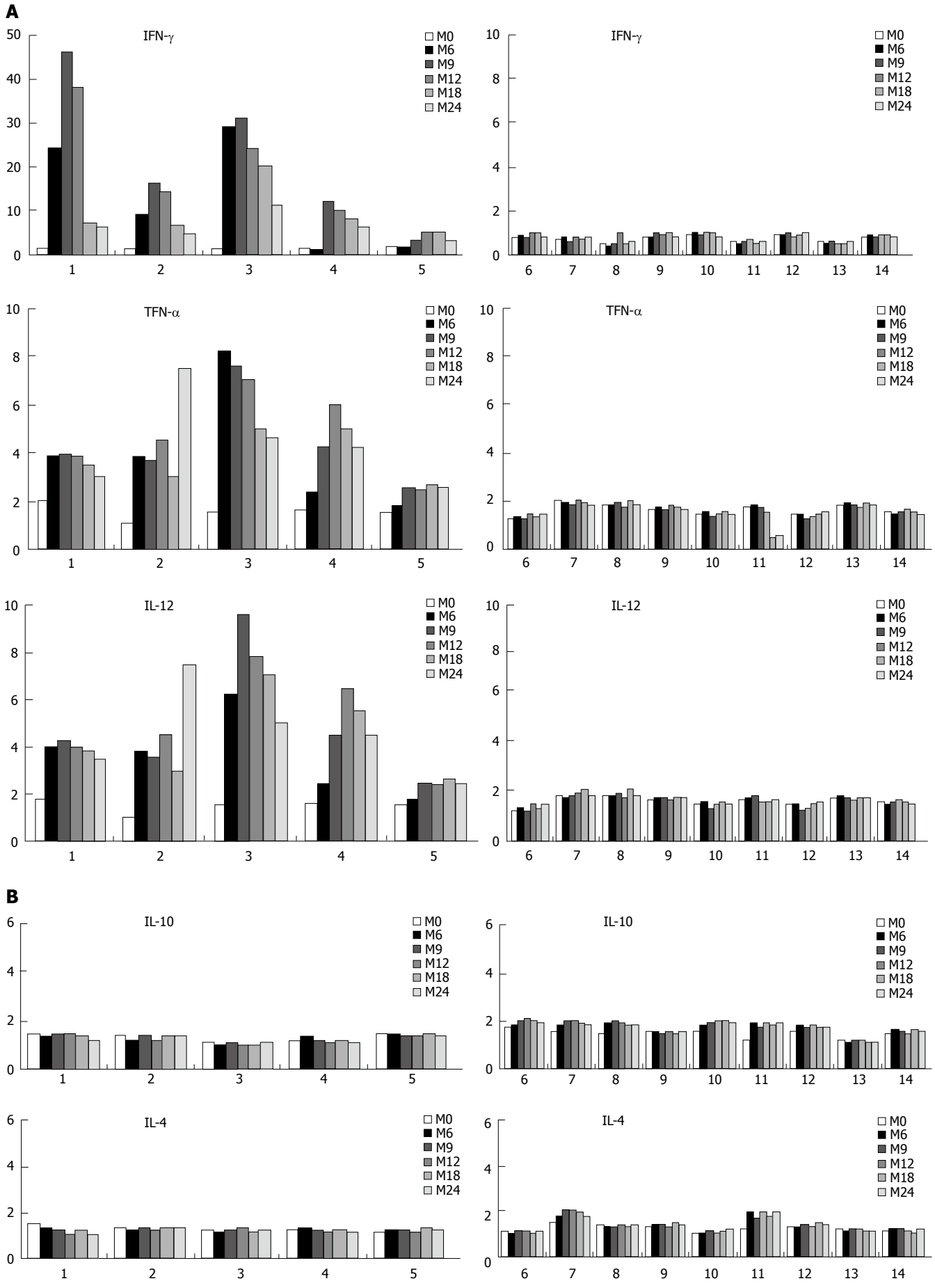

Figure 2 Cytokine production levels in PBMCs in combination therapy patients. The vertical axis represents the ratio of the mean cytokine concentration of triplicate cultures obtained in the presence of antigen to that obtained without antigen. The numbers in the horizontal lines represent the patients. **A:** Th1 cytokine production in PBMCs in combination therapy patients; **B:** Th2 cytokine production in PBMCs in combination therapy patients.

HBV replication in some patients, since serum HBV DNA levels were significantly lower at 12 and 24 mo in responders who showed HBV-antigen-specific IFN-γ, TNF-α and IL-12 production *in vitro* in PBMCs that was augmented in responders. The production of HBV-antigen-specific Th1 (IFN-γ, TNF-α, and IL-12) and Th2 (IL-4 and IL-10) cytokines did not increase, or remained unchanged in non-responders. Cytokine production showed a Th1-like pattern, as well as induction of PBMCs, and was consistent with the results of Rico *et al*[18].

CD4+ T cells are necessary for the maintenance of the effector functions of CD8+ T cells during chronic viral infection[20]. Activated CD8+ cytotoxic T cells can kill virus-infected cells by utilizing both perforin-dependent and Fas-mediated cytotoxic mechanisms[21]. CD8+ T cells can also secrete anti-viral cytokines such as IFN-γ and TNF-α[22]. The HBV-antigen-specific T-cell reactivity observed in our study may be relevant for the outcome of the infection, because it may be crucial to provide help to CD8+ cytotoxic T-cell responses to lyse and clear HBV-infected cells[23-27]. Nevertheless, eradication of HBV may be accomplished by other cells non-cytolytically by transcription and replication of HBV[28,29]. Based on our results, it is not possible to establish the mechanism contributing to HBV clearance (in this study we did not investigate CD8+ cytotoxic T cells in the cytotoxic assay). However, the decrease in serum HBV DNA levels was almost coincident with the increase of IFN-γ production by antigen-specific CD4+ T cells and was not preceded by an increase in serum ALT levels (as represented by patient 2, data not shown). These results suggest that cytotoxic T cells are unlikely to contribute to the control of HBV replication in combination therapy with ribavirin and IFN-α; results that are supported by those of Rico *et al*[18]. CD4+ T cells appear to directly participate in the anti-viral response (by producing anti-viral cytokines) rather than indirectly (by helping cytotoxic T cells) in this combination therapy of ribavirin and IFN-α. A role for Th1 cells in controlling viral infection is supported by experiments showing that they can clear influenza[30,31] and vaccinia virus[32] infections in a cytotoxic-T-lymphocyte-independent manner. Further, a direct, cytokine-dependent anti-viral role for CD4+ T cells, which produce Th1 cytokines (Th1 cells), has been shown in HBV transgenic mice[33,34]. These reports support our concept that the increased production of anti-viral cytokines by PBMCs plays a crucial role in the control of HBV replication in combination therapy with IFN-α and ribavirin. This combination therapy for chronic hepatitis B not only significantly reduced viremia levels but also induced lasting CD4+ T-cell proliferation and Th1 cytokine release at the site of infection, which may have led to sustained HBV eradication, as suggested by Rico *et al*[18]. Further studies will be needed to ascertain whether the anti-viral mechanism of combination therapy is by a route different from the one normally employed.

In conclusion, the present study indicated that combination therapy with ribavirin and IFN-α for anti-HBe-positive patients significantly reduces viremia, and induces CD4+ T-cell proliferation and Th1 cytokine secretion in patients with chronic hepatitis B.

COMMENTS

Background

Some recent reports have suggested that ribavirin shows antiviral and immune effects against various infectious diseases, including hepatitis B and C. It is suggested that combination therapy with IFN-α plus ribavirin for chronic hepatitis B significantly reduces viremia; however, the mechanisms involved remain unclear.

Research frontiers

Previous studies have suggested that combination therapy with IFN-α plus ribavirin for chronic hepatitis B significantly reduces viremia; however, the mechanism is unclear. In the present study, we investigated the anti-viral mechanism of combination therapy with IFN-α and ribavirin against chronic hepatitis B by analyzing T-cell proliferative responses in patients and determining Th1 cytokine levels.

Innovations and breakthroughs

In this study, we analyzed HBV-specific CD4+ T-cell proliferative responses and determined Th1 cytokine levels in PBMCs. Our results indicated that combination therapy of patients with chronic hepatitis B with ribavirin and IFN-α significantly reduced viremia, and induced CD4+ T-cell proliferation and Th1 cytokine secretion.

Applications

This study indicates that combination therapy with ribavirin and IFN-α for chronic hepatitis B significantly reduces viremia; thus, this combination may represent an alternative treatment option to achieve sustained eradication of HBV in patients with chronic hepatitis B refractory to IFN-α treatment.

Peer review

This is an interesting report of combination therapy with IFN-α plus ribavirin against chronic hepatitis B. The results suggest that HBV-antigen-specific CD4+ T cells may directly control HBV replication and secretion of anti-viral Th1 cytokines by PBMCs, utilizing combination therapy with ribavirin and IFN-α against chronic hepatitis B.

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