行政院國家科學委員會補助專題計畫成果報告

計畫名稱：C型肝炎病毒核心抗原特異性細胞毒殺性T細胞在致病機轉所扮演的角色(III)

計畫類別：整合型計畫

計畫編號：NSC 89-2315-B-002-019-MH

執行日期：民國八十八年八月一日至民國八十九年七月三十一日

計畫主持人：江伯倫 教授

計畫參與人員：孫昭玲 小姐

執行單位：台大醫學院臨床醫學研究所

中文摘要

本研究計畫的主要目的是要探討細胞毒殺性T細胞在C型肝炎病毒感染中所扮演的角色，我們分別利用人類的自然感染和動物模式來加以探討。在人類的研究發現，我們已經建立能夠表現C型肝炎核心蛋白的Hep G2細胞，由於Hep G2細胞本身可以表現人類HLA-A2的分子，所以我們便利用能夠HLA-A2分子的HCV患者來進行研究。結果發現這些HCV患者在接受干擾素合併ribavirin治療後可以增加第一型T輔助細胞、自然殺手細胞和細胞毒殺性T細胞的活性。由前一年得到的結果，我們更進一步研究ribavirin可能的機轉，於是我們在動物身上進行相關的研究，結果發現ribavirin的確可以增強第一型T輔助細胞的活性及細胞毒殺性T細胞的活性。同時，利用體內的研究和試管內的研究也看到ribarivin能夠有效地增加IL-12的製造，而我們知道IL-12是增加第一型T輔助細胞的活性，而ribavirin可能因爲增加IL-12的製造而達到促進細胞毒殺性T細胞的活性，而達到治療C型肝炎病毒的效果。

關鍵詞：C型肝炎，細胞毒殺性T細胞

Abstract

Combination IFN-α and ribavirin therapy of hepatitis C virus infected patients has been reported to improve the response rate to 50%. Meanwhile, the previous study also demonstrated that it enhanced the natural killer cell activity and the level of hepatitis C virus specific IFN-g in the patients treated with ribavirin and IFN-α. To further study the role of ribavirin in hepatitis C virus-specific immune responses, we immunized mice with hepatitis C virus core protein with or without ribavirin. Forty days after immunization, we found that the mice immunized with core antigen once every two weeks and 0.5 mg ribavirin every day showed higher level of core-specific IgG2a as compared with those mice immunized with core antigen only. In addition, we found that *in vitro* recalled core antigen increased the levels of the T helper type 1 cytokines produced by spleen cells. In addition, the lipopolysaccharide (LPS)-stimulated peritoneal cells produced higher level of IL-12 in ribavirin-treated mice. The percentage of CD3+ cells increased significantly both in spleen cells and peritoneal cells. Both the percentage and activity of natural killer cells were enhanced dramatically. The core-specific cytotoxic T cell activity also increased significantly. Thus, ribavirin may significantly promote the T helper type 1 immune response *in vivo*, furthermore, the effect of ribavirin on IL-12 level produced by LPS-stimulated peritoneal cells may contribute to the Th1 enhancing effect.

Key words: Hepatitis C virus, cytotoxic T cells, ribavirin

計畫目標：

成果報告：

本年度的研究計畫是進一步研究干擾素合併ribavirin治療的效果，我們爲了更進一步研究ribavirin對免疫機能調節的機轉，於是我們便將ribavirin合併C型肝炎核心蛋白注射入小鼠體內，再去追蹤小鼠體內相關的免疫反應變化。結果我們發現在小鼠體內的第一型T輔助細胞的活性，包括IgG2a抗體、自然殺手細胞活性和細胞毒殺性T細胞增加。而這些活性的增加將是有效清除體內C型肝炎病毒重要的因素，所以本研究進一步確定ribavirin的效果。同時，本研究還進一步研究其中的機轉。結果發現ribavirin可以增加IL-12的分泌，而IL-12的產生又跟第一型T輔助細胞的活性有著密切的關係。本研究結果的發現對了解干擾素合併ribavirin的治療機轉和未來研發新的藥物有著相當重要的影響。也是因爲如此，本研究結果已被接受發表於J. Hepatology，即將在今年十一月發表。隨報告附上論文的初稿。

Journal of Hepatology 2000: 33: 000–000
Printed in Denmark · All rights reserved
Munksgaard · Copenhagen

Journal of Hepatology
ISSN 0168-8278

Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T helper cell response correlates with the increased IL-12 level

Shih-Hua Fang[1,4], Lih-Hwa Hwang[3], Ding-Shinn Chen[3] and Bor-Luen Chiang[1,2]

[1]*Graduate Institute of Microbiology,* [2]*Graduate Institute of Clinical Medicine,* [3]*Hepatitis Research Center, College of Medicine, National Taiwan University, Taipei, and* [4]*Department of Medicine, China Medical College, Taichung, Taiwan*

Backgrounds/Aims: **Combination IFN-α and ribavirin therapy for hepatitis C virus-infected patients has been reported to improve the response rate up to 50%. In this study, we aimed to study further the role of ribavirin in hepatitis C virus-specific immune responses.**

Methods: **We immunized mice with hepatitis C virus core protein with or without different concentrations of ribavirin. Forty days after immunization, hepatitis C virus-specific immune responses were followed in these mice.**

Results: **We found that the mice immunized with core antigen once every 2 weeks and 0.5 mg ribavirin every day showed higher levels of core-specific IgG2 compared with those mice immunized with core antigen only. In addition, core antigen-stimulated spleen cells produced higher levels of T helper type 1 cytokines and the core-specific cytotoxic T cell activity also increased significantly. Furthermore, lipopolysaccharide-stimulated peritoneal cells produced higher levels of IL-12 in ribavirin-treated mice, and peritoneal cells isolated from naïve mice also produced significantly higher level of IL-12 when cultured with ribavirin.**

Conclusions: **Ribavirin may significantly promote the T helper type 1 immune response *in vivo*, and, furthermore, the effect of ribavirin on IL-12 level produced by accessory cells may contribute to the T helper type 1 enhancing effect.**

Key words: **Hepatitis C virus; IL-12; Ribavirin; Type 1 T helper cell.**

RIBAVIRIN, a guanosine analogue, is active against various RNA and DNA viruses, including influenza viruses (1,2), respiratory syncytial viruses (RSV) (3,4), Lassa fever (5) and murine immunodeficient virus (6). It has been evaluated as therapy for chronic hepatitis C virus (HCV) infection (7). Combining ribavirin with interferon (IFN)-α proved more effective for the treatment of chronic hepatitis C than IFN-α alone (8). Meanwhile, combination therapy has also induced a sustained biochemical and virological response in a significant proportion of patients with IFN-α-resistant chronic hepatitis C (9). When ribavirin was evaluated as a monotherapy for chronic hepatitis C, it improved or normalized alanine transaminase (ALT) levels during 6- and 12-month courses of treatment. However, the response was usually transient, and no changes in HCV viremia could be seen with ribavirin monotherapy (10,11). In addition, similar results were also obtained when ribavirin was used to treat hepatitis B virus (HBV) infection (12). The observations prompted some investigators to postulate that ribavirin works as an immunomodulatory agent, as opposed to an antiviral agent (13–15). Earlier studies have shown that low concentrations (0.1 to 1 μg/ml) of ribavirin moderately enhance B cell responses, whereas slightly higher concentrations (5 to 10 μg/ml) abolish the functions of B cells (16). Recent studies have shown that the beneficial effects of ribavirin are mediated by the inhibition of induction of macrophage pro-inflammatory cytokines and the production of IL-4 by Th2 cells, whereas it did not diminish the production of IFN-γ in Th1 cells (13). In HCV NS3-immunized mice, the levels of Ag-specific IgG2a and IgG2b increased markedly (14). We have earlier observed that the type 1 T helper cell related immunological changes in patients with chronic hepa-

Received 2 December 1999; revised 11 April; accepted 28 April 2000

Correspondence: Bor-Luen Chiang, Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, No. 1, Chang-Teh Street, Taipei, Taiwan.
Tel: 2 23970800 ext. 7299 or 7302. Fax: 2 23972031.
e-mail: gicmbor@ ha.mc.ntu.edu.tw

titis C receiving IFN-α combined with ribavirin may contribute to better sustained responses (data not shown).

To further study the possible mechanisms of ribavirin on HCV-specific immune responses, we analyzed the Ag-specific humoral and cellular immune responses in mice after immunization with HCV core protein and different doses of ribavirin.

Materials and Methods

Animals

Female BALB/c mice were obtained from and maintained in the Animal Center of the College of Medicine, National Taiwan University. All mice used were 10 weeks of age. The animal room was on a 12-h light and 12-h dark cycle with a constant temperature of 25°C (±2°C) and humidity maintained. Each group consisted of 6 mice.

Ribavirin and HCV core protein

Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) was obtained (as Virazole) from ICN Pharmaceuticals (Irvine, CA, USA) and was dissolved in PBS prior to use. HCV core protein (C190) was produced in BL21 cells and purified by denaturing nickel column (Fang et al., unpublished data).

Cell

YAC-1 and Balb/c 3T3 were obtained from American Type Culture Collection (Rockville, MD, USA). All cells were cultured with complete RPMI-1640 medium containing 10% fetal calf serum, antibiotics, L-glutamine, and HEPES buffer.

Immunizations and in vivo *ribavirin treatment*

Ribavirin was given intraperitoneally (i. p.) in 100 μl PBS for 40 days starting 2 days prior to immunization. Groups A to E mice were given a daily dose: 1, 0, 1, 0.5, 0.1 mg ribavirin, respectively. Groups B to E mice were also injected with 25 μg core protein in 100 μl Freund's complete adjuvant intraperitoneally on day 0 and then boosted with 50 μg of core protein in 100 μl Freund's incomplete adjuvant on days 14 and 28. Group A mice were injected with ribavirin plus PBS as the adjuvant-only control.

Antibody ELISA

Blood was obtained from the retro-orbital venous plexus on day −2 (pre-immunization), day 14, and day 28 after immunization with core protein. The serum was collected and stored at −20°C before further assay. Anti-core IgG1 and IgG2a were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (17). The plates were read in a microplate autoreader at 450 nm. The antibody levels of samples were compared with standard. The standard serum was a pool of serum collected from core-immunized mice which had had strong responses. The concentration of standard serum was arbitrarily assigned 1 ELISA unit (EU).

Core antigen-specific proliferation assay

The mice were sacrificed on day 40 and spleen cells were harvested. For proliferation assay, spleen cells were plated in microplates at the concentration of 10^5 cells per well in the presence or absence of 0.0625 μg/ml core protein for 5 days with the addition of 1 μCi [^{3}H] thymidine (TdR; Amersham) for the last 16 h. The labeled cells were harvested on cellulose filters, quenched, and the level of [^{3}H] TdR incorporation was determined by a liquid scintillation β-counter. The result of core-specific proliferative response was expressed as stimulation index (S.I.). An S.I. value over 3 was defined as positive.

Cytokine assay

To determine the concentrations of IL-2, IFN-γ, IL-4, and IL-5, 5×10^6 spleen cells from groups A to E mice were incubated with or without 0.25 μg/ml of core antigen in 24-well flat-bottomed plates for 24 h. The culture supernatant was collected and analyzed by using a sandwich-ELISA according to the manufacturer's instructions. Standards were prepared from recombinant mouse IL-2, IFN-γ, IL-4, or IL-5 separately (PharMingen, San Diego, CA, USA). The sensitivity of IL-2, IFN-γ, IL-4, or IL-5 is 781 pg/ml, 390 pg/ml, 97.6 pg/ml, or 39 pg/ml, respectively.

To determine the concentrations of IL-6, IL-10, and IL-12, 4×10^5 peritoneal cells from groups A to E mice were incubated with or without 10 μg/ml LPS (Sigma) for 24 h. For *in vitro* study, peritoneal cells from naive mice (4×10^5 cells/ml) were incubated in the presence of 0, 16, 62.5, 250, 1000 pg/ml ribavirin, respectively, with or without 2.5 μg/ml LPS (Sigma) for 24 h. Spleen cells from naive mice were stimulated with anti-CD3 (PharMingen, San Diego, CA, USA) plus anti-CD28 (PharMingen, San Diego, CA, USA) in the presence of 20 ng/ml IL-2 for 2 days. After washing, 5×10^6 spleen cells were incubated with 0, 0.25, 0.5, 1, 2 ng/ml of IL-12 in 24-well flat-bottomed plates for 24 h. The culture supernatant was collected and analyzed as described above. Standards were prepared from recombinant mouse IL-6, IL-10, or IL-12 separately (PharMingen, San Diego, CA, USA). The sensitivity of IL-6, IL-10, or IL-12 is 781 pg/ml, 781 pg/ml, or 195 pg/ml, respectively.

Phenotypic analysis of spleen cells

Surface staining was performed by incubating 5×10^5 spleen cells with phycocrythrin [PE]-conjugated anti-CD19 and fluorescein isothiocyanate [FITC]-conjugated anti-CD3, PE-conjugated anti-CD4 and FITC-conjugated anti-CD3, PE-conjugated anti-CD8 and FITC-conjugated anti-CD3, PE-conjugated anti-CD69 and FITC-conjugated anti-CD3, PE-conjugated anti-DX-5 and FITC-conjugated anti-CD3 (PharMingen, San Diego, CA, USA). All antibody incubations were performed at 4°C for 30 min. The cells were washed and resuspended in 0.5 ml of PBS, 0.1% sodium azide and subjected to FACScan analysis. A total of 10000 cells was counted and the frequency of each cell surface marker was determined using appropriate software (FACScan, Becton Dickinson, Mountain View, CA, USA). The flow cytometry was regularly calibrated with CaliBRITE beads (Becton Dickinson, Mountain View, CA, USA).

Natural killer cell cytotoxicity assay

Spleen cells were incubated with 1×10^4 ^{51}Cr-labeled YAC-1 cells in 96-well round-bottomed microtiter plates at an effector/target (E/T) ratio of 10, 50, and 100, respectively. The cytotoxic activity was performed in the standard 4-h ^{51}Cr-release assay. Specific lysis was calculated as follows: ^{51}Cr released (in percentage)=(E-S)/(T-S)×100, where E is the amount of chromium released in the presence of effector cells, S is the spontaneous release of the label in media alone, and T is the total amount of chromium released in the presence of 50% HCl.

Preparation of core-expressed target cells for cytotoxic T cell activity assay

To clone the core gene, primers C190-N (5'-AAG AAT TCC GTG CAC CAT GAG CAC GAA T-3') and C190-C (5'-AAG TCG ACC TAA GCG GAA GCT GGG AT-3') were used to perform polymerase chain reaction. The resulting DNA fragment was sequenced and found to be identical to the sequences reported in GenBank. The cDNA obtained was digested with *EcoRI* and *Sal I* and cloned into a retroviral vector, S2 (18). The resulting construct, C190/S2, was transfected into GP+E-86 packaging cell line (19) by the calcium phosphate precipitation method. The supernatant was collected 2 days later and used to infect GP+AM12 amphotropic packaging cell line (20). Virus-producing clones were obtained after selection of the transduced GP+AM12 cells by G418 (0.8 μg/ml). Viruses were harvested 16 to 18 h after fresh medium was placed onto the virus-producing cells, and were used to infect Balb/c 3T3 cells in the presence of 8 μg/ml of polybrene for 24 h. Transfected Balb/c 3T3 cells were selected by G418 (0.4 μg/ml) until resistant clones appeared. All of the G418-resistant clones were pooled to check the expression of core protein by Western blot.

Core-specific cytotoxic T cell activity assay

Spleen cells (10^7 cells/ml) were plated on a 24-well plate and stimulated with irradiated 10^6 syngenic stimulator cells, C190/Balb/c 3T3, for 48 h and replaced with complete RPMI-1640 medium plus 100 U of rIL-2 per ml for another 3 days. *In vitro*-stimulated spleen cells were collected to perform cytotoxicity assay against ^{51}Cr-labeling C190/Balb/c 3T3 or Balb/c 3T3 cells as control at an E/T ratio of 12.5, 25, and 50, respectively. The cytotoxic activity was performed by standard 4-h ^{51}Cr-releasing assay as described.

Statistical analysis

Data are presented as mean±SEM. The data analyzed by paired Student's *t*-test, and a *p*-value of 0.05 or less was considered to be statistically significant.

Results

C190-specific antibody responses of mice treated with different doses of ribavirin

To investigate the effect of ribavirin, the mice were divided into five groups: A to E. Group A mice received only 1 mg ribavirin everyday, without core protein. Groups B to E mice were immunized with core antigen once every 2 weeks and received 0, 1, 0.5, or 0.1 mg ribavirin every day, respectively. No significant difference in the body weight among these groups indicated that the immunization dose and schedule did not affect the normal growth of those mice.

To determine the C190-specific humoral immune responses, the sera on day 2 prior to immunization and on days 14, 28, and 35, were collected and analyzed for C190-specific antibodies by ELISA. C190-specific total IgG levels were increased on days 28 and 35 after immunization (data not shown). Furthermore, we analyzed the level of anti-core IgG1 and IgG2a among all groups. The data suggest that the level of IgG2a antibody was significantly higher in groups C to E when compared to control and C190-only immunized mice (Table 1, $p<0.01$). This suggests that ribavirin may promote T helper type 1 response *in vivo*.

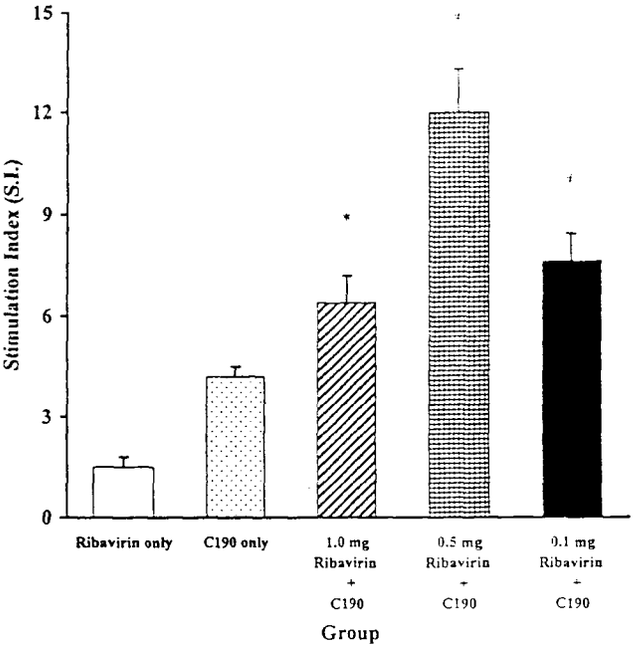

*Fig. 1. C190-specific proliferative response of spleen cells. Spleen cells were plated at 10^5 cells in the presence or absence of 0.0625 μg/ml core protein for 5 days with the addition of 1 μCi [^{3}H] thymidine (TdR; Amersham) for the last 16 h. Stimulation index was calculated by the cpm value of each experiment over that of cell only. *represents p<0.05 compared to group B. #represents p<0.01 compared to group B.*

HCV core protein-specific proliferative responses

When the C190-specific antibody reached the plateau, the mice were killed on day 40, and the core-specific

TABLE I

The levels of IgG1 and IgG2a produced in different groups of mice

Group	IgG1				IgG2a			
	Day −2	14	28	35	Day −2	14	28	35
A	0.01±0.01#	0.01±0.02	0.02±0.02	0.02±0.03	0.02±0.01	0.03±0.02	0.02±0.02	0.02±0.01
B	0.03±0.02	0.11±0.05	0.28±0.08	0.41±0.09	0.05±0.02	0.07±0.04	0.13±0.05	0.19±0.07
C	0.02±0.03	0.13±0.04	0.29±0.09	0.51±0.07	0.01±0.02	0.14±0.07*	0.19±0.08	0.22±0.04
D	0.02±0.01	0.14±0.04	0.27±0.04	0.54±0.06	0.01±0.01	0.13±0.04*	0.23±0.02*	0.53±0.03**
E	0.02±0.01	0.11±0.05	0.29±0.02	0.52±0.04	0.02±0.02	0.11±0.01	0.19±0.01	0.38±0.05**

Group A received 1 mg ribavirin every day.
Group B received C190 protein once every second week.
Group C received 1 mg ribavirin every day and C190 protein once every 2 weeks.
Group D received 0.5 mg ribavirin every day and C190 protein once every 2 weeks.
Group E received 0.1 mg ribavirin every day and C190 protein once every 2 weeks.
#represents ELISA unit: Mean±SEM.
*$p<0.05$ compared to that of group B at the same time point.
**$p<0.01$ compared to that of group B at the same time point.

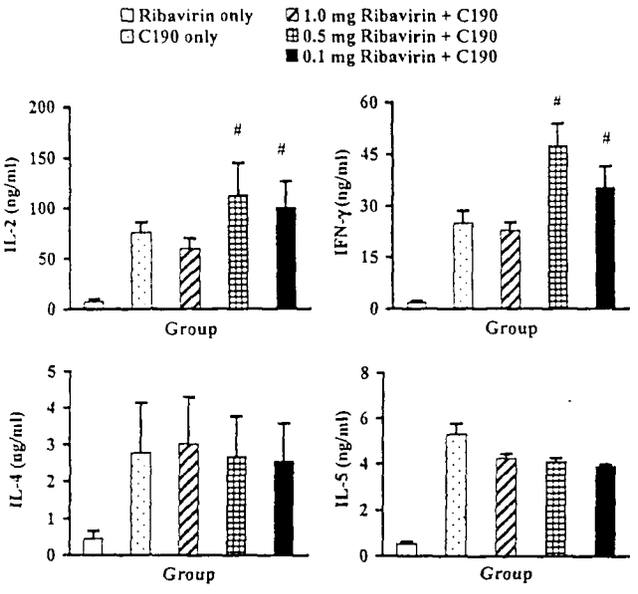

Fig. 2. Cytokines produced by spleen cells from different groups recalled in vitro *with core antigen. 5×10^6 spleen cells were incubated with or without 0.25 μg/ml of core antigen in 24-well plates for 24 h. The culture supernatants were collected and analyzed using a sandwich-ELISA as described in Methods. #represents* $p<0.01$ *compared to group B.*

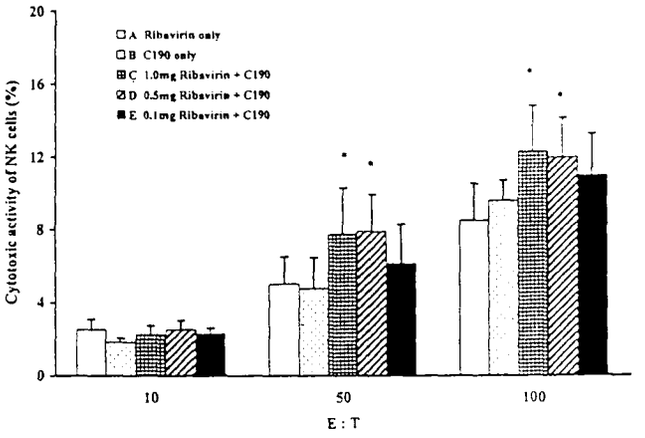

*Fig. 3. NK cell activity of different groups of mice. Spleen cells were incubated with 1×10^4 ^{51}Cr-labeled YAC-1 at an effector/target (E/T) ratio of 10, 50, and 100, respectively. The cytotoxic activity was performed in the standard 4-h ^{51}Cr-release assay. *represents* $p<0.05$ *compared to group B.*

cellular immune response was analyzed. The results show that the stimulation index in groups C to E was higher than that in group B (Fig. 1, group C, $p<0.05$; groups D and E, $p<0.01$).

Cytokine profiles produced by core-stimulated spleen cells

To further understand the cytokine profiles, the cytokines produced by the C190-stimulated spleen cells *in vitro* were assayed. The levels of Th1 cytokines, IL-2 and IFN-γ, were significantly increased in groups D and E, as compared to those in group B (Fig. 2, $p<0.01$). Although the levels of Th2-related IL-5 tended to decrease in mice treated with ribavirin, it was not statistically significant. There was no difference in the levels of IL-4 among these five groups.

Phenotypic analysis of spleen cells and peritoneal cells

We found that the percentage of total T cells increased significantly both in spleen cells of groups C to E (Table 2) and in peritoneal cells of group D ($p<0.01$, data not shown). Meanwhile, the percentage of NK cells also increased significantly in the spleen cells of groups A, C to E; all the mice had received ribavirin treatment. (Table 2, $p<0.05$).

TABLE 2

The percentage of different cell types in spleen cells of different treated groups of mice

Group	Cell type				
	Total B cell	Total T cell	CD4+ T cell	CD8+ T cell	NK cell
A	48.05±2.04#	34.17±1.09	20.55±2.22	15.43±2.07	4.89±0.25*
B	45.05±1.48	36.56±2.04	20.96±1.11	17.61±1.48	2.01±0.41
C	46.81±1.56	41.56±2.47*	21.32±1.09	19.34±2.11	4.24±0.89*
D	45.23±1.84	47.84±3.14**	28.79±2.64**	18.92±1.45	3.81±0.15*
E	47.01±2.48	45.02±2.07*	23.55±1.66	19.12±0.94	3.27±0.57*

Group A received 1 mg ribavirin every day.
Group B received C190 protein once every second week.
Group C received 1 mg ribavirin every day and C190 protein once every 2 weeks.
Group D received 0.5 mg ribavirin every day and C190 protein once every 2 weeks.
Group E received 0.1 mg ribavirin every day and C190 protein once every 2 weeks.
#: represents ELISA unit: Mean±SEM.
*$p<0.05$ compared to that of group B at the same time point.
**$p<0.01$ compared to that of group B at the same time point.

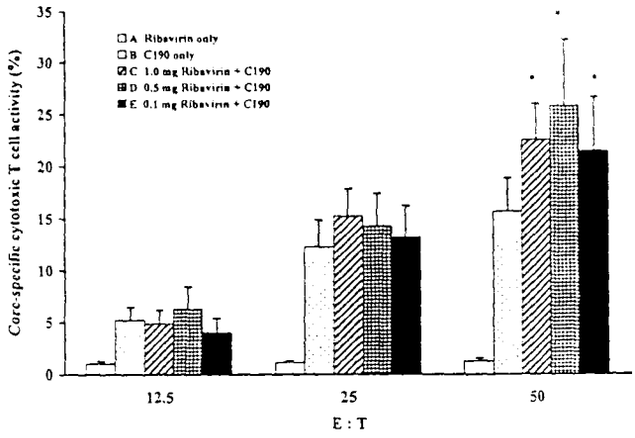

*Fig. 4. Core-specific cytotoxic T cell activities in different groups of mice. Spleen cells were stimulated with irradiated stimulator cells, C190/Balb/c 3T3, and were collected to perform cytotoxicity assay against ^{51}Cr-labeling C190/Balb/c 3T3 or Balb/c 3T3 cells as control at an E/T ratio of 12.5, 25, and 50, respectively. The cytotoxic activity was performed by standard 4-h ^{51}Cr-releasing assay as described in Methods. Data was given as means of the lysis % of C190/Balb/c 3T3 minus that of control Balb/c 3T3 cells. *represents $p<0.05$ compared to group B. #represents $p<0.01$ compared to group B.*

Cytolytic activity of natural killer cells and core-specific cytotoxic T lymphocytes

To further determine the effect of ribavirin on HCV-specific cellular immune responses, both NK cell activity and C190-specific cytotoxic response were monitored by standard 4-h ^{51}Cr-releasing assay. We found that the NK cell activities increased in groups C to E (Fig. 3, $p<0.05$), and the cytotoxic T cell activities also improved in groups C to E (Fig. 4, groups C&E, $p<0.05$; group D, $p<0.01$). This suggests that ribavirin may enhance C190-specific and non-specific cellular immunity, which has been thought to be correlated with Th1 activity.

IL-12 produced by LPS-stimulated peritoneal cells of ribavirin-treated mice

Antigen-presenting cells play a very important role in inducing immune responses and many of these cells are found in the peritoneal cavity. We found higher IL-12 levels in LPS-stimulated peritoneal cells of groups A, C to E, which had all received ribavirin (Fig. 5, $p<0.01$). The enhancement of IL-12 level seemed to be correlated with ribavirin but not with core antigen immunization.

In vitro *effect of ribavirin on IL-12 production by peritoneal cells*

To study further the effect of ribavirin on the cytokine production of peritoneal cells, peritoneal cells of naïve mice were incubated with different concentrations of

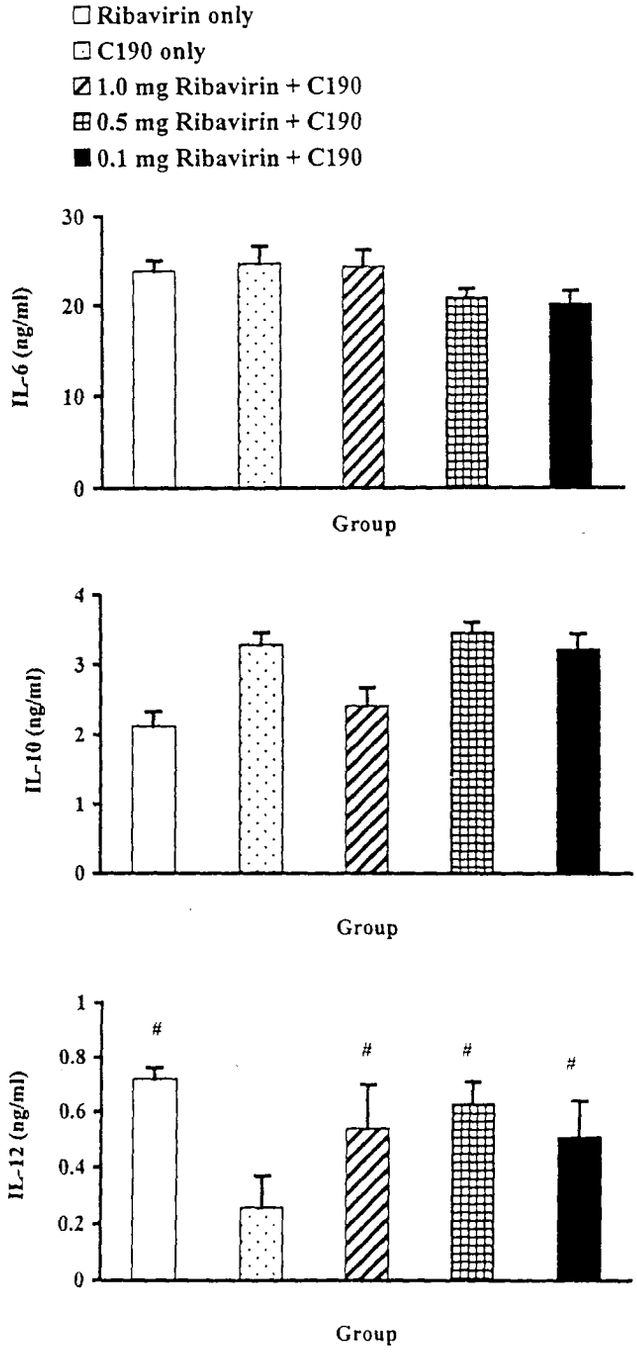

Fig. 5. Cytokine produced by LPS-stimulated peritoneal cells from different concentrations of ribavirin-treated mice. Peritoneal cells (4×10^5 cells/ml) were incubated without or with 10 μg/ml LPS for 24 h. The culture supernatants were collected and analyzed as described in Methods. #represents $p<0.01$ compared to group B.

ribavirin (0, 0.016, 0.0625, 0.25, 1 ng per ml) *in vitro* with or without LPS stimulation. The data suggested that IL-12 levels increased significantly in the presence of ribavirin, especially with the lower concentration of ribavirin (Fig. 6A, $p<0.01$). To understand the effect of ribavirin-induced IL-12 on the cytokine production profiles of spleen cells, different concentrations of IL-12 were added to stimulated spleen cells and found that IL-12 promoted the secretion of Th1 type cytokines, IFN-γ and IL-2 (Fig. 6B, $p<0.01$ & C, $p<0.05$). These results suggest that ribavirin may increase IL-12 production by accessory cells and subsequently modulate the Th1 responses.

Discussion

Previous reports have demonstrated that ribavirin has an inhibitory effect on human peripheral blood mononuclear cell mitogenesis (21), and also reduces the growth of mouse lymphoma cells up to 50% of the control (22). Ribavirin not only inhibits the growth of lymphocytes, it also directly affects the functions of some immune cells. It has been shown that ribavirin may modulate the plaque-forming cell (PFC) responses (16). Furthermore, ribavirin inhibited viral-induced macrophage production of the pro-inflammatory cytokines and Th 2 cytokine profiles (13), and also mast cell mediator release (23). In addition, ribavirin inhibited not only the replication of respiratory syncytial virusess (RSV) in respiratory epithelial cells, but also decreased the IL-6 level and subsequent acute-phase responses (24). Taken together, the results suggest that ribavirin may modulate the immune responses. However, the mechanism has yet to be clarified. In the treatment course of HCV-infected patients, they received orally 1200 mg ribavirin daily (20 mg/kg/day), in this study, the dose of ribavirin (5–50 mg/kg/day) applied to the mice was modified according to another study in a murine AIDS model (6).

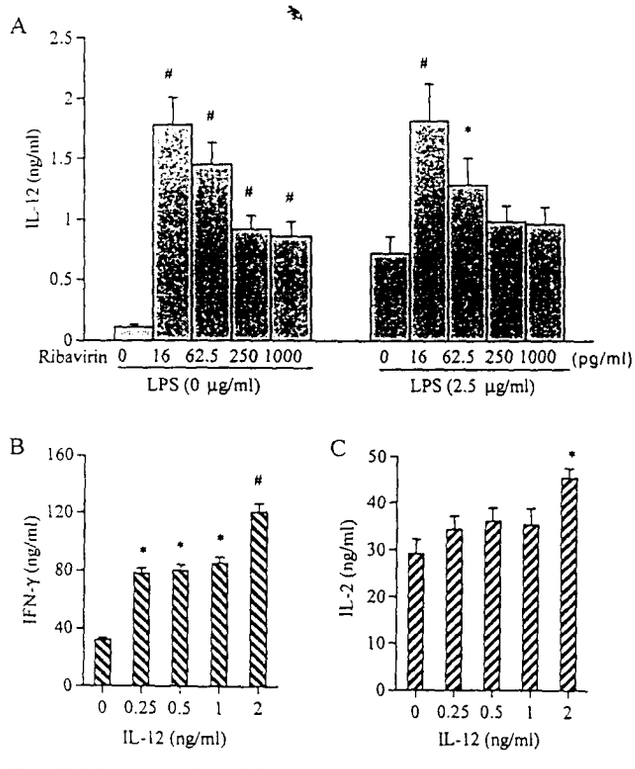

*Fig. 6. IL-12 produced by peritoneal cells cultured with different concentrations of ribavirin (A) and the effect of IL-12 on the cytokine profiles of spleen cells (B, C). Peritoneal cells (4×10^5 cells/ml) from naive mice were incubated in the presence of 0, 16, 62.5, 250, 1000 pg/ml ribavirin, respectively, with or without 2.5 μg/ml LPS for 24 h. *represents $p<0.05$ compared to that without ribavirin. #represents $p<0.01$ compared to that without ribavirin (A). Anti-CD3 antibody stimulated spleen cells (5×10^6 cells/ml) were incubated with 0, 0.25, 0.5, 1, 2 ng/ml of IL-12 for 24 h. *represents $p<0.05$ compared to that without IL-12. #represents $p<0.01$ compared to that without IL-12 (B, C).*

The T helper (Th) subsets are largely defined by unique patterns of cytokine secretion such that Th1 cells produce IL-2, IFN-γ and tumor necrosis factor, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (25). The differences in cytokine secretion account for the distinct functions of Th1 cells, which mediate cellular immunity, and Th2 cells, which enhance humoral immunity. Previous studies had shown that the activation of Th2-like responses in acute hepatitis C patients correlates with chronic evolution, whereas the result in cases with predominant Th1-like responses is recovery (26,27). In the group of mice treated with core antigen once every 2 weeks and 0.5 mg ribavirin every day, we found that serum levels of C190-specific IgG2a (Th1-like) increased significantly compared to those of the other groups. Consistent with a shift toward a Th1-like pattern, we demonstrated that C190-stimulated spleen cells secrete higher levels of IL-2 and IFN-γ in the mice immunized with core antigen plus ribarivin. Most interestingly, both C190-specific cytotoxicity and the NK activity also increased dramatically with the addition of ribavirin.

To further understand the possible mechanism of ribavirin, the cytokine profile of antigen-presenting cells was assayed. Among the data, it was noted that the level of IL-12 produced by LPS-stimulated peritoneal cells increased in mice treated with ribavirin. IL-12, a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) KDa (28) encoded by two separate genes, is produced mainly by monocytes, macrophages, and other antigen-presenting cells in response to stimulation by a variety of

microorganisms (29–31). IL-12 exerts multiple biological activities mainly through T and natural killer cells by inducing their production of IFN-γ, which augments their cytotoxicities, and by enhancing their proliferation potential. In addition, IL-12 promotes the differentiation of CD4+ naïve T cells into Th1 cells and thereby plays a central role in the regulation of the Th1/Th2 balance (32,33). Previous studies have shown that IL-12 may be useful as an immunotherapeutic agent for many pathogens, such as virus and bacteria infection (34–37). All the evidence suggested IL-12 is closely correlated with the development of Th1 activity. In this study, increased IL-12 levels produced by peritoneal residual cells were found in mice receiving ribavirin treatment. Most importantly, the increased IL-12 level seemed to be correlated with ribavirin treatment but not core antigen immunization.

We found that naïve peritoneal cells cultured with ribavirin *in vitro* produced significant level of IL-12. Moreover, IL-12 can increase IFN-γ and IL-2 produced by anti-CD3 antibody-stimulated spleen cells. Consistent with the previous studies, IL-12 induces Th1 type cytokines produced by naïve T cells in murine and human system (38–40). It is emphasized that the mouse model here is not representative of HCV infection, but rather of HCV-specific immune prophylaxis. However, the data suggested that ribavirin may promote C190-specific immune responses towards a Th1-like pattern by inducing the antigen-presenting cells to produce a higher IL-12 level. This interesting finding needs more studies to elucidate the immunoregulatory mechanism of ribavirin and provide help in exploring more efficient preventive and therapeutic management for HCV patients.

Acknowledgements

This research was supported by a grant, NSC 89–2315–002–005-MH from the National Science Council, Republic of China. We would also like to thank Liver Disease Prevention & Treatment Research Foundation for providing the fellowship to S. H. Fang.

References.

1 Gillbert BE, Wilson SZ, Knight V, Couch RB, Quarless JM, Dure I, et al. Ribavirin small-partical aerosol treatment of infections caused by influenza virus strain A/Victoria/7/83 (H1N1) and B/Texas/a/84. Antimicrob Agents Chemother 1985; 27: 309–13.

2. Wray SK, Gilbert BE, Knight V. Effect of ribavirin triphosphate on primer generation and elonglation during influenza virus transcription *in vitro*. Antiviral Res 1985; 5: 39–48.
3. Hall CB, McBride JT, Walsh EE, Bell DM, Gala CL, Hildreth S, et al. Aerosolized ribavirin treatment of infants with respiratory syncytial viral infection. A randomized double-blind study. N Engl J Med 1983; 308: 1443–7.
4. McIntosh K, Kurachet SC, Cairns LM, Burns JC, Goodspeed B. Treatment of respiratory viral infection in an immunodeficient infant with ribavirin aerosol. Am J Dis Child 1984; 138: 305–8.
5. McCormick JB, King IJ, Webb PA, Scribner CL, Craven RB, Johnson KM, et al. Lassa fever. Effective therapy with ribavirin. N Engl J Med 1986; 314: 20–6.
6. Omar RF, Harvie P, Gourde P, Desormeaux A, Tremblay M, Beauchamp D, et al. Antiviral efficacy and toxicity of ribavirin and foscarnet each given alone or in combination in the murine AIDS model. Toxicol Applied Pharmacol 1997; 143: 140–51.
7. Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. Lancet 1991; 337: 1058–61.
8. Lai MY, Kao JH, Yang PM, Wang JT, Chan KW, Chu JS, et al. Long-term efficacy of ribavirin plus interferon alpha in the treatment of chronic hepatitis C. Gastroenterology 1996; 111: 1307–12.
9. Brillanti S, Garson J, Foli M, Whitby K, Deaville R, Masci C, et al. A pilot study of combination therapy with ribavirin plus interferon alfa for interferon alfa-resistant chronic hepatitis C. Gastroenterology 1994; 107: 812–7.
10. Dusheiko G, Main J, Thomas HR, Reichard O, Lee C, Dhillon A, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. J Hepatol 1996; 25: 591–8.
11. Bodenheimer HC, Lindsay KL, Davis GL, Lewis JH, Thung SN, Seeff LB. Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. Hepatology 1997; 26: 473–7.
12. Fried MW, Fong TL, Swain MG, Park Y, Beames MP, Banks SM, et al. Therapy of chronic hepatitis B with a 6-month course of ribavirin. J Hepatol 1994; 21: 145–50.
13. Ning Q, Brown D, Parodo J, Cattral M, Gorczynski R, Cole E, et al. Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. J Immunol 1998; 160: 3487–93.
14. Hultgren C, Milich DR, Weiland O, Sallberg M. The antiviral compound ribavirin modulates the T helper (Th) 1/Th2 subset balance in hepatitis B and C virus-specific immune responses. J Gen Virol 1998; 79: 2381–91.
15. Tam RC, Pai B, Bard J, Lim C, Averett DR, Phan UT, et al. Ribavirin polarizes human T cell responses toward a type I cytokine profile. J Hepatol 1999; 30: 376–82.
16. Powers CN, Peavy DL, Knight V. Selective inhibition of functional lymphocyte subpopulations by ribavirin. Antimicrob Agent Chemo 1982; 22: 108–14.
17. Ou-Yang P, Chiang BL, Hwang LH, Chen YG, Yang PM, Chi WK, et al. Characterization of monoclonal antibodies against hepatitis C virus nonstructural protein 3: different antigenic determinants from human B cells. J Med Virol 1999; 57: 345–50.
18. Hsieh CL, Chen BF, Wang CC, Liu HH, Chen DS, Hwang LH. Improved gene expression by a modified bi-cistronic retroviral vector. Biochem Biophys Res Commun 1995; 214: 910–7.
19. Markowitz D, Goff S, Bank A. A safe packaging line for gene transfer: Separating viral gens on two different plasmids. J Virol 1998; 62: 1120–4.
20. Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. Virol 1998b; 167: 400–6.
21. Heagy W, Crumpacker C, Lopez PA, Finberg RW. Inhibition of immune functions by antiviral drugs. J Clin Invest 1991; 87: 1916–24.
22. Lee HJ, Pawlak K, Nguyen BT, Robins RK, Sadee W. Biochemical differences among four inosinate dehydrogenase inhibitors, mycophenolic acid, ribavirin, tiazofurin, and selenazofurin, studied in mouse lymphoma cell culture. Cancer Research 1985; 45: 5512–20.
23. Marquardt DL, Gruber HE, Walker LL. Ribavirin inhibits mast cell mediator release. J Pharm Exp Ther 1987; 240: 145–9.
24. Jiang Z, Kunimoto M, Patel JA. Autocrine regulation and ex-

perimental modulation of interleukin-6 expression by human pulmonary epithelial cells infected with respiratory syncytial virus. J Virol 1998; 72: 2496–9.
25. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136: 2348–57.
26. Diepolder HM, Zachoval R, Hoffmann RM, Wierenga EA, Santantonio T, Jung MC, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995; 346: 1006–7.
27. Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo G. Detection of Th2-like T helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology 1997; 25: 449–58.
28. Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cell type 1 and cytotoxic lymphocytes. Blood 1994; 84: 4008–27.
29. Benjamin D, Sharma V, Kubin M, Klein JL, Sartori A, Hollday J, et al. Il-12 expression in AIDS-related lymphoma B cell lines. J Immunol 1996; 156: 1626–37.
30. Kang K, Kubin M, Cooper KD, Lessin SR, Trinchieri G, Rook AH. IL-12 synthesis by human Langerhans cells. J Immunol 1996; 156: 1402–7.
31. Lawrence C, Nauciel C. Production of interleukin-12 by murine macrophages in response to bacterial peptidoglycan. Infect Immun 1998; 66: 4947–9.
32. Seder RA, Kelsall BL, Jankovic D. Differential roles for IL-12 in the maintenance of immune responses in infectious *versus* autoimmune disease. J Immunol 1996; 157: 2745–8.
33. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. Adv Immunol 1998; 70: 83–243.
34. Cleric M, Lucey DR, Berzofsky JA, Pinto LA, Wynn TA, Blatt SP, et al. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 *in vitro*. Science 1993; 262: 1721–4.
35. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murrhy K. Development of Th1 CD4+ T cells through UL-12 produced by *Listeria*-induced macrophages. Science 1993; 260: 547–9.
36. Afonso LCC, Scharton TM, Vieira LQ, Wysocka M, Trinchieri G, Scott et al. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science 1994; 263: 235–7.
37. Williams NJ, Harvey JJ, Duncan I, Booth RFG, Knight SC. Interleukin-12 restores dendritic cell function and cell-mediated immunity in retrovirus-infected mice. Cell Immunol 1998; 183: 121–30.
38. Puddu P, Fantuzzi L, Borghi P, Varano B, Rainaldi G, Guillemard E, et al. IL-12 induces IFN-γ expression and secretion in mouse peritoneal macrophages. J Immunol 1997; 159: 3490–7.
39. McKnight AJ, Zimmer GJ, Fogelman I, Wolf SF, Abbas AK. Effects of IL-12 on helper T cell-dependent immune responses *in vivo*. J Immunol 1994; 152: 2172–9.
40. Moller DR, Forman JD, Liu MC, Noble PW, Greenlee BM, Vyas P, et al. Enhanced expression of IL-12 associated with Th1 cytokine profiles in active pulmonary sarcoidosis. J Immunol 1996; 156: 4952–60.