Journal of General Virology (1998), **79**, 2381–2391. Printed in Great Britain

The antiviral compound ribavirin modulates the T helper (Th)1/Th2 subset balance in hepatitis B and C virus-specific immune responses

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Ribavirin is effective in combination therapies against chronic hepatitis C virus (HCV) infection, although its direct antiviral properties are unclear. We therefore studied the immune-modulatory effects of ribavirin on hepatitis B virus (HBV)- and HCV-specific immune responses. During a 24 week placebo-controlled ribavirin trial in ten patients with chronic HCV infection, HCV antibodies and alanine aminotransferase (ALT) levels decreased transiently whereas the serum levels of HCV RNA remained stable. Effects of ribavirin on human and murine phytohaemagglutinin (PHA)-activated T cells included inhibition of *in vitro* proliferation and modulation of IL-2, IL-4, IFN-γ and TNF-α levels. HBcAg- and HBeAg-specific IL-2 and IFN-γ levels were $\geqslant$ 25-fold higher in mice immunized with HBV core- or e-antigens (HBcAg, HBeAg) while receiving ribavirin compared to untreated mice, but IL-4 and IL-6 remained constant. Concordantly, a slight shift was observed in the IgG subclass distribution of the humoral responses of ribavirin-treated mice to HBeAg and HCV NS3 protein. Ribavirin treatment of HBeAg-transgenic (HBeAg-Tg) mice induced a dose-dependent down-regulation of T helper (Th)2-mediated antibody production to HBeAg. In ribavirin-treated HBeAg-Tg mice anti-HBe IgG1 (positively regulated by Th2 cytokines) decreased simultaneously as both anti-HBe IgG2a (positively regulated by Th1 cytokines) levels and *in vitro* T-cell IFN-γ production increased, indicating a change in the Th1/Th2 balance. Thus, the present data suggest that ribavirin is not strictly an antiviral compound, but rather it alters the T-cell balance in the immune system.

Introduction

The guanosine analogue ribavirin has shown antiviral effects on a number of viruses *in vitro* (Hosoya *et al.*, 1993; Shigeta *et al.*, 1992). The major action of ribavirin seems to be inhibition of inositol monophosphate dehydrogenase leading to depletion of the cell pool of GTP (Agbaria *et al.*, 1994; Balzarini *et al.*, 1991). Despite the *in vitro* inhibitory effect of ribavirin on many viruses, the beneficial clinical effect of ribavirin treatment for some viral infections including HCV has not been fully elucidated (Spanish Ribavirin Trial Group, 1991; Ribavirin ARC Study Group, 1993; American Academy of Pediatrics Committee on Infectious Diseases, 1996; Japour *et al.*, 1996). Attempts have been made to assess the efficacy of ribavirin for the treatment of infection with respiratory syncytial virus (RSV) and chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (Di Bisceglie *et al.*, 1992, 1995; Reichard *et al.*, 1991).

Ribavirin has profound anti-proliferative effect on human peripheral blood mononuclear cells (PBMCs) *in vitro*: doses ranging from 1 to 10 μM are effective in inhibiting mitogen-induced proliferation (Heagy *et al.*, 1991). This anti-proliferative effect of ribavirin may result from depletion of GTP pools, which are essential for proliferating T cells (Fairbanks *et al.*, 1995). The *in vitro* inhibitory levels are within the same range as the steady-state levels achieved in serum during ribavirin treatment of humans (Connor *et al.*, 1993; Englund *et al.*, 1990).

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In childhood RSV and influenza virus infections, ribavirin treatment seems mainly to reduce the symptoms of infection (Rodriguez *et al.*, 1994). In murine models, the major symptoms during RSV infection seem to be mediated by T helper (Th)2-like immune responses, which are enhanced by prior immunization, similar to observations from studies on humans (Connors *et al.*, 1994; Srikiatkhachorn & Braciale, 1997; Waris *et al.*, 1996). Interestingly, it was recently shown that ribavirin has an inhibitory effect on the production of IL-6 in pulmonary epithelial cells infected with RSV (Jiang *et al.*, 1998).

During ribavirin treatment of chronic HBV infection, serum levels of HBV DNA tend to decrease (Fried *et al.*, 1994; Kakumu *et al.*, 1993). However, in most cases the virus load rebounds on cessation of therapy (Fried *et al.*, 1994). Some patients show reduced alanine aminotransferase (ALT) levels during treatment but again rebound is seen on cessation of treatment (Fried *et al.*, 1994). In contrast, ribavirin treatment of chronic HCV infection has very little effect on the virus load (Di Bisceglie *et al.*, 1995; Dusheiko *et al.*, 1996), despite the fact that most treated patients will normalize ALT levels transiently during treatment (Di Bisceglie *et al.*, 1992, 1995; Dusheiko *et al.*, 1996; Reichard *et al.*, 1991). These data suggest that the beneficial effect of ribavirin in combination with IFN-α (Lai *et al.*, 1996; Schvarcz *et al.*, 1995; Reichard *et al.*, 1998) in the treatment of chronic HCV infection may be immune-mediated and not directly antiviral.

To study the immune-modulatory mechanisms of ribavirin further we analysed cytokine production by polyclonally activated T cells in the presence of ribavirin *in vitro*. We also analysed the immune effects of ribavirin *in vivo* in murine models. Studies on mice transgenic for hepatitis B e-antigen (HBeAg) have shown that antibodies to HBeAg (anti-HBe) can be induced by activation of endogenous HBeAg-specific T cells through injection of an HBeAg-derived synthetic T-cell site (Milich *et al.*, 1990, 1991). It is well-documented that the anti-HBe seroconversion in these mice is mediated by HBeAg-specific Th2-like immune responses (Milich *et al.*, 1995*a*, *b*; Hultgren *et al.*, 1997). Therefore, this model has been useful in analysing the effects of potentially immune-modulatory compounds *in vivo* (Milich *et al.*, 1995*c*).

Methods

■ **Human serum samples.** Forty serum samples were obtained from a total of ten patients who had received either placebo or ribavirin for 24 weeks (Dusheiko *et al.*, 1996). Samples were obtained at weeks zero, 12, 24 and 48. At each time-point the levels of ALT and HCV RNA were determined. The levels of HCV RNA were quantified by both limiting dilution and a commercial assay (Branched DNA 1st Generation, Chiron Corp.). Informed consent was obtained from all study subjects. The study protocol was approved by the local ethical research committee at Huddinge University Hospital.

■ **Human PBMC.** Fresh PBMCs were obtained from volunteer donors and were separated by gradient centrifugation as described previously (Zhang *et al.*, 1997*b*).

■ **Mice.** Inbred B10 (H-2^b), B10.S (H-2^s), B10 × B10.S_{F1} (H-2^{bxs}) and BALB/c (H-2^d) mice were obtained from either the breeding facility at The Scripps Research Institute or BK Universal, Sollentuna, Sweden. All mice were used at 6–12 weeks of age. All animal experiments followed approved guidelines and were approved by the local ethical research committee on animal research (no. S100/96).

B10 × B10.S_{F1} (H-2^{bxs}) mice heterozygous for the HBeAg transgene have been described previously (Milich *et al.*, 1990). Seroconversion from HBeAg to antibodies against HBeAg (anti-HBe) can be induced in these mice by immunization with a synthetic H-2^b-restricted T-cell site corresponding to residues 129–140 of the HBc/eAg sequence (Milich *et al.*, 1987, 1991). This anti-HBe seroconversion is mediated by an HBeAg-specific Th2-like cell population (Hultgren *et al.*, 1997; Milich *et al.*, 1995*b*).

■ **Ribavirin and antigens.** Ribavirin was obtained (as Virazole) from ICN Pharmaceuticals and was dissolved in either PBS or tissue culture medium prior to use. Phytohaemagglutinin (PHA) was purchased from Sigma. Full-length recombinant HBcAg (rHBcAg) encompassing residues 1–183 was produced in *E. coli* as previously described (Schödel *et al.*, 1993). This protein assembles into 27-nm-diameter particles. A truncated protein lacking the 34 carboxy-terminal residues of HBcAg which is monomeric at pH 9·6 (HBeAg-9·6) exhibits HBeAg antigenicity when used as a solid-phase antigen (Milich *et al.*, 1988). An additional recombinant form of HBeAg contained nine residues of pre-core and the first 150 residues of HBcAg and was designated PCe (Schödel *et al.*, 1993).

E. coli-derived recombinant HCV core and NS3 proteins (Jin & Peterson, 1995; Jin *et al.*, 1995) were kindly provided by Darrell L. Peterson, Commonwealth University, VA, USA.

A synthetic peptide corresponding to residues 129–140 of the HBcAg sequence (p129–140) (Milich *et al.*, 1987) was produced by solid-phase peptide synthesis based on 9-fluorenylmethoxycarbonyl protection on the α-amino group using automated procedures (Milligen 9050 Plus, Millipore).

■ **Enzyme immunoassays (EIAs).** Human total IgG and IgG subclass distribution of HCV-specific antibodies were determined by EIA using recombinant HCV core and NS3 proteins as previously described (Zhang *et al.*, 1997*b*).

Total IgG and IgG subclass distribution of murine anti-HBc and anti-HBe antibodies were determined as previously described using rHBcAg or HBeAg-9·6 as the solid-phase ligands at 5 μg/ml and 1 μg/ml, respectively (Milich *et al.*, 1995*a*; Sällberg *et al.*, 1993).

■ **Tissue culture media.** Click's medium was used for proliferation and cytokine analysis of lymph node T cells and was prepared with 0·5 % mouse serum, L-glutamine, vitamins and 50 μM 2-mercaptoethanol (2-ME) as described (Milich *et al.*, 1987). The analysis of splenic T cells was performed in RPMI 1640 (GibcoBRL) containing 5 % foetal bovine serum, L-glutamine, HEPES, sodium pyruvate, penicillin–streptomycin and 2-ME.

■ **Polyclonal activation of human and murine T cells.** Polyclonal activation of human and murine T cells was performed by plating 5×10^5 murine splenocytes, lymph node cells or human PBMCs per well in microplates. The *in vitro* effects of ribavirin were analysed by culturing cells with 1 μg PHA and a dilution series from 205 to 0·026 μM ribavirin for 48 h at 37 °C. Analysis of T-cell proliferation and cytokine production was then performed as described below.

■ **Immunizations and *in vivo* ribavirin treatment.** Ribavirin was given intraperitoneally (i.p.) in 100 μl PBS for 14 to 24 days starting

3 days prior to immunizations. Mice were given daily doses ranging from 0 to 1·5 mg per day. Groups of ribavirin-treated or untreated mice were then injected i.p. or subcutaneously (s.c.) with 10 μg rHBcAg, 50 μg PCe or 50 μg HCV NS3, emulsified in 100 μl Freund's complete adjuvant.

The HBeAg-Tg mice were treated as described above with the exception that they were immunized with the synthetic T-cell site p129–140. Mice were bled by retro-orbital puncture on a weekly basis for 6 weeks.

■ **Proliferation and cytokine assays.** Nine to eleven days after immunization the mice were sacrificed and draining lymph node cells or spleen cells were harvested (Sällberg *et al.*, 1996, 1997; Zhang *et al.*, 1997*a*). Single-cell suspensions were prepared in either Click's medium or in RPMI 1640 and were plated in microplates at 6×10^5 cells per well for proliferation and cytokine assays. Supernatants were removed at 24 and 48 h for determination of IL-2, IL-4, IL-5, IL-6, IL-10 and IFN-γ. For measuring T-cell proliferation the plates were incubated for 72 to 96 h with the addition of 1 μCi [^{3}H]thymidine (TdR; Amersham) for the last 16 h. The labelled cells were harvested on cellulose filters, quenched, and the level of [^{3}H]TdR incorporation was determined by a liquid scintillation β-counter.

The presence of cytokines in culture supernatants was determined as previously described (Milich *et al.*, 1995*c*; Sällberg *et al.*, 1997). In brief, the presence of human and murine IL-2 in supernatants was determined by proliferation of the IL-2-sensitive NK′A cell line (Milich *et al.*, 1987) and by species-specific EIAs (Endogen or Biosource). Presence of human and murine IL-4 was determined by proliferation of the IL-4-sensitive CT4.S cell line (Milich *et al.*, 1995*a*) and by species-specific EIAs (Endogen or Biosource). The presence of human and murine IL-5, IL-6, IL-10, IL-12 and IFN-γ was determined by species-specific sandwich EIAs (Pharmingen, Endogen or Biosource) (Sällberg *et al.*, 1997; Zhang *et al.*, 1997*b*). All commercial EIAs were performed according to the manufacturer's instructions. Results from representative experiments are presented.

■ **Quantitative cytokine mRNA RT–PCR.** Human IL-4 and TNF-α mRNA were quantified with a commercial kit (Biosource). In brief, 2×10^6 cells were cultured in the absence or presence of 5 μg PHA and different concentrations of ribavirin for 24 h in 1 ml RPMI 1640 medium. One μg total RNA, extracted with TRIzol reagent (GibcoBRL), was transcribed to cDNA using M-MLV reverse transcriptase (GibcoBRL) and oligo(dT_{12-18}) according to the manufacturer's recommendation. The cDNA was amplified together with an internal quantification-standard DNA with *Taq* polymerase (Promega) and cytokine- and standard DNA-specific primers. The amplified DNA was then quantified spectophotometrically according to the manufacturer's instructions.

Results

Effects of ribavirin on HCV RNA and on humoral HCV-specific and non-specific responses in humans

In the patient group receiving placebo no changes in levels of antibodies to HCV core or NS3 occurred during therapy (Fig. 1 *a–b*). In contrast, five of the six patients receiving ribavirin showed a transient decrease in HCV-core antibody levels during treatment ($P < 0{\cdot}01$, Fisher's exact test), and four of five patients with HCV NS3 antibodies showed a decrease in NS3-specific IgG during treatment. Thus, ribavirin seems to affect humoral responses during chronic HCV infection. No significant changes were noted in the IgG subclass distribution of anti-HCV core or NS3 during therapy (data not shown).

Most importantly, the mean levels of serum HCV RNA did not change significantly during ribavirin or placebo treatment compared with pre-treatment levels (not significant, Mann–Whitney). No differences in HCV RNA levels were noted between the two treatment regimens of patients with chronic HCV infection, except that HCV RNA levels in the ribavirin treatment group were slightly higher than those in the placebo group in the 48 week samples (Fig. 1 *c*; $P < 0{\cdot}05$, Mann–Whitney).

ALT levels decreased significantly during weeks 12 to 24 in the group receiving ribavirin (Fig. 1*d*; $P < 0{\cdot}01$, Mann–Whitney) but rebounded at week 48. No changes in ALT levels were noted in the placebo group.

In all samples the serum levels of IL-4, IL-12 and IFN-γ were determined by commercial EIAs. We did not observe any significant changes, or differences, in serum cytokines within or between the treatment groups (Fig. 1*e*, and data not shown). Thus, ribavirin treatment did not affect the levels of serum cytokines significantly.

The present data reiterate perfectly the previously noted transient ALT decrease in the presence of constant levels of HCV RNA during ribavirin treatment (Di Bisceglie *et al.*, 1995; Dusheiko *et al.*, 1996). They are representative of the *in vivo* effects of ribavirin in chronic HCV infection, and suggest strongly that the transient decrease in HCV-specific antibodies during ribavirin treatment occurred independently of the virus load.

Effects of ribavirin on PHA-induced proliferation and cytokine production

The effects of ribavirin on PHA-induced human and murine T-cell proliferation and cytokine production were determined. Corroborating previous observations (Heagy *et al.*, 1991), we noted that ribavirin inhibited PHA-induced proliferation of both human and murine T cells at 1 μM (Fig. 2*a*, and data not shown).

The effect of ribavirin on PHA-induced, human PBMC-derived IL-4 (Th2-like) and TNF-α (Th1-like) mRNA expression levels was determined in 24 h *in vitro* cultures. Ribavirin (100 μg/ml) reduced the PHA-induced IL-4 mRNA copy number from 4341 to 553 per 40 000 cells. In contrast, the same dose of ribavirin increased the PHA-induced TNF-α mRNA copy number from 48 063 to 196 610 per 80 000 cells. Thus, ribavirin seemed to alter cytokine mRNA expression in human PBMCs.

Ribavirin inhibited all cytokine production in PHA-activated murine T cells at concentrations over 100 μM (Fig. 2*b–d*). However, murine IL-2, IL-4 and IFN-γ production were inhibited at 8·2, 8·2 and 41 μM ribavirin, respectively. This suggests that cytokine production is less sensitive to ribavirin inhibition than is cell proliferation. Collectively, these data suggest that a difference in sensitivity may exist between Th1-

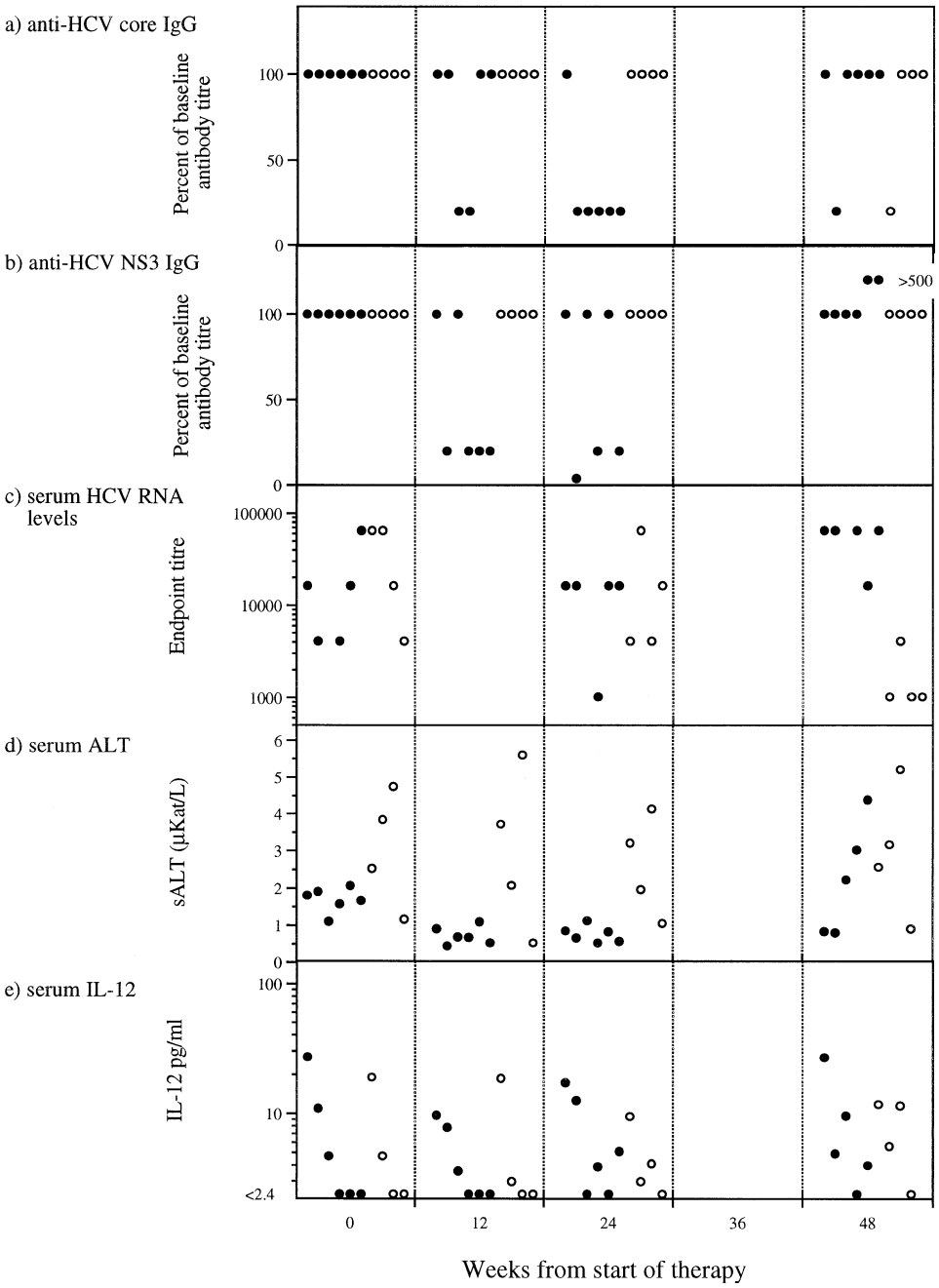

Fig. 1. Changes in HCV core- (*a*) and NS3- (*b*) specific antibody titres, serum HCV RNA (*c*) measured by limiting dilution, serum ALT (sALT) (*d*) and serum IL-12 (*e*) during a 24 week placebo (○) or ribavirin (●) treatment. Antibody levels are given as percentages of pre-treatment levels of HCV-specific antibodies. The pre-treatment titres of HCV core antibodies ranged from 2160 to 77760 whereas the HCV NS3 antibody titres ranged from 360 to 77760. HCV RNA levels are given as the endpoint titre, sALT as μKat/l (1 U/l = 0·017 Kat/l) and IL-12 as pg/ml.

like and Th2-like cytokine responses to ribavirin in both human and mouse systems.

Effects of ribavirin on HBc/eAg-specific proliferation and cytokine production

The effect of ribavirin treatment of mice immunized with HBcAg or HBeAg on *in vivo* priming of splenic T cells was analysed further. Mice were given daily doses of 100 μl PBS containing 0 or 1·5 mg ribavirin for a total of 14 days starting 3 days before immunization (day −3). Interestingly, in the HBcAg-immunized and ribavirin-treated group a profound increase in IL-2 and IFN-γ production was noted, evidenced by

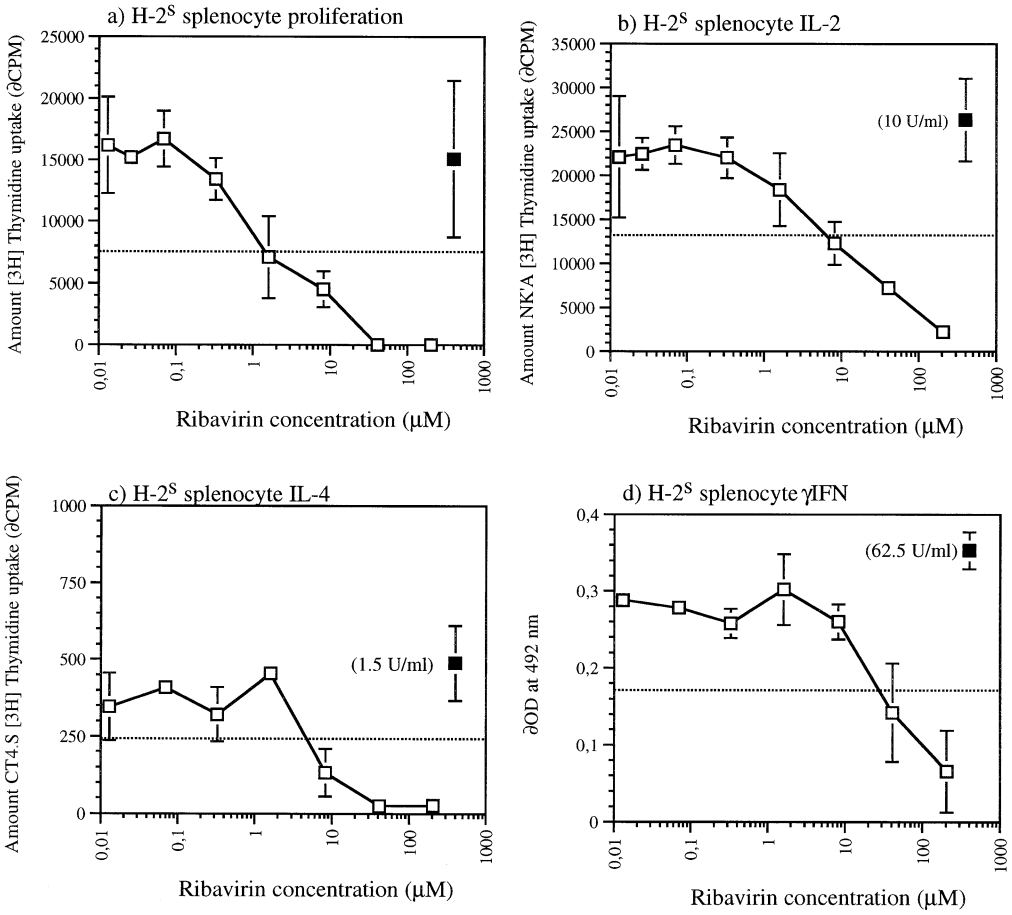

Fig. 2. Ribavirin inhibition of PHA-induced (2 μg/ml) murine splenocyte *in vitro* proliferation (*a*) and IL-2 (*b*), IL-4 (*c*) and IFN-γ (*d*) levels. Proliferation was determined at 72 h, IL-2 at 24 h, and IL-4 and IFN-γ at 48 h. Data are given as means $\pm$SD of duplicate samples minus the mean values for spontaneous proliferation. ■, Means $\pm$ SD of controls treated with PHA alone. ....., 50% of PHA controls.

a leftward shift of the antigen dilution curves (Fig. 3*a*, *g*). T cell IL-2 production in the ribavirin-treated mice was induced by *in vitro* recall at 25-fold lower HBcAg concentrations (0·008 μg/ml vs 0·2 μg/ml; Fig. 3*a*), and T cell IFN-γ production was induced in the ribavirin-treated mice at 125-fold lower HBcAg concentrations (0·00032 μg/ml vs 0·04 μg/ml; Fig. 3*g*), compared to untreated mice. Also, in the HBeAg-immunized mice, *in vitro* recall did not induce detectable IFN-γ in the untreated mice, whereas T cells from the ribavirin-treated mice produced IFN-γ at HBeAg concentrations of $\geqslant$ 1 μg/ml (Fig. 3*h*). No major differences were observed in the endpoint titrations of HBcAg or HBeAg with respect to *in vitro* recall of IL-4 and IL-6, although at high antigen concentrations IL-6 was produced at higher levels in the ribavirin-treated groups (Fig. 3*c–f*).

Additionally, similar experiments were performed on lymph node-derived T cells from mice immunized with HBcAg. Groups of mice (B10 × B10.S_{F1}) immunized with HBcAg were given daily doses of 100 μl PBS containing 0 or 1·0 mg ribavirin for 14 days. Mice were sacrificed 9 to 11 days after immunization, and draining lymph nodes were harvested and analysed by *in vitro* recall using HBcAg. Compared to untreated mice, the ribavirin-treated group showed lower levels of HBcAg-induced IL-4 ($<$ 0·9 U/ml vs 1·9 $\pm$ 0·5 U/ml) and higher levels of HBcAg-induced IFN-γ (190 $\pm$ 10 U/ml vs 70 $\pm$ 20 U/ml). Altogether, these data suggest that ribavirin alters the Th1/Th2 balance *in vivo*.

In vivo effects of ribavirin treatment on HBV- and HCV-specific humoral responses in wild-type mice

It has been shown that both HBeAg and HCV NS3 prime a polyclonal T-cell response consisting of both Th1 and Th2 cells (Hultgren *et al.*, 1997; Milich *et al.*, 1997; Sällberg *et al.*, 1996, 1997). We therefore analysed the effect of ribavirin treatment during the priming of a polyclonal HBeAg- or NS3-specific immune response in wild-type mice. Since we did not observe any significant effect of daily doses of 1·5 mg ribavirin on the total humoral HBeAg- and NS3-specific immune responses at day 14, the ribavirin therapy was continued for a

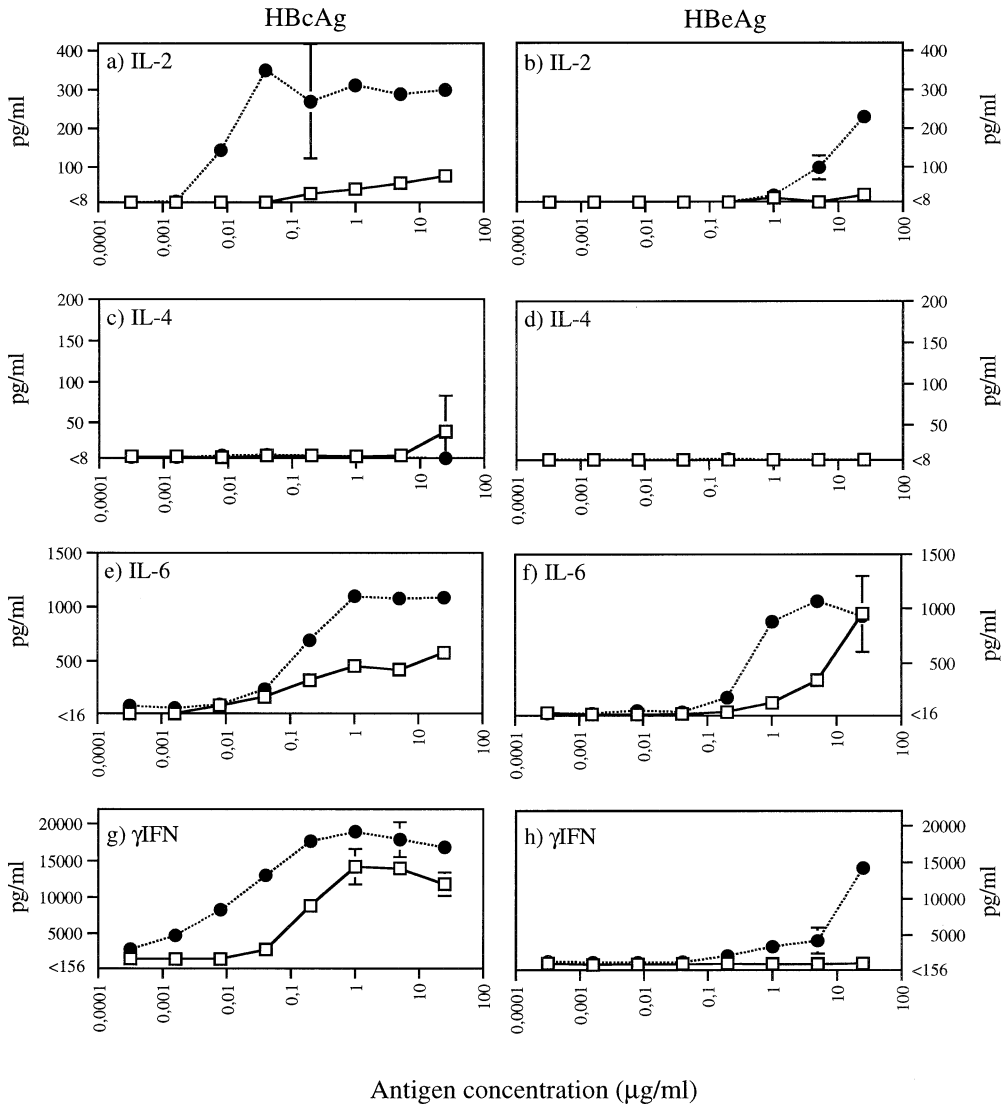

Fig. 3. Effects of daily PBS (□) or 1·0 mg ribavirin (●) treatment (days −3 to 10) of BALB/c mice immunized at day 0 with HBcAg (*a*, *c*, *e* and *g*) or HBeAg (*b*, *d*, *f* and *h*) on *in vitro* recall with HBcAg and HBeAg. HBcAg- and HBeAg-induced cytokine production were determined as described in Methods. Values have been converted to pg/ml using standard dilutions of the respective cytokine.

further 2 weeks (Fig. 4*a–b*). However, 1 week after cessation of ribavirin therapy no differences were seen in the total IgG levels between the treatment groups. In both HBeAg- and HCV NS3-immunized mice small differences were seen in the IgG subclass distribution of the ribavirin-treated mice, with the most marked increases in IgG2a and IgG2b (Fig. 4*d–e*). Some conclusions can be drawn from this experiment. First, ribavirin is not directly immune-suppressive on B-cell activation and proliferation. Second, ribavirin does not seem to have a profound effect on the B-cell responses promoted by a mixed Th1/Th2 population. Third, since the Th1- and Th2-like subsets both promote humoral responses through different cytokines, the absence of a total suppression of antibody production shows that ribavirin is not generally immune-suppressive but is somewhat selective, as evidenced by the shift in IgG subclass distribution (Fig. 4*d–e*).

In vivo effects of ribavirin treatment on an HBeAg-specific Th2-mediated immune response

To evaluate further the possible *in vivo* effects of ribavirin treatment on a Th2-mediated immune response, groups of HBeAg-Tg mice, immunized with an HBeAg-derived synthetic T-cell site, were given daily doses of 100 μl PBS containing 0, 0·75, 1·0 or 1·5 mg ribavirin for 14 days.

In the mice receiving PBS alone, anti-HBe seroconversion occurred after 2 weeks (Fig. 4*c*). In contrast, a dose-dependent inhibition of the anti-HBe response was seen in the ribavirin-

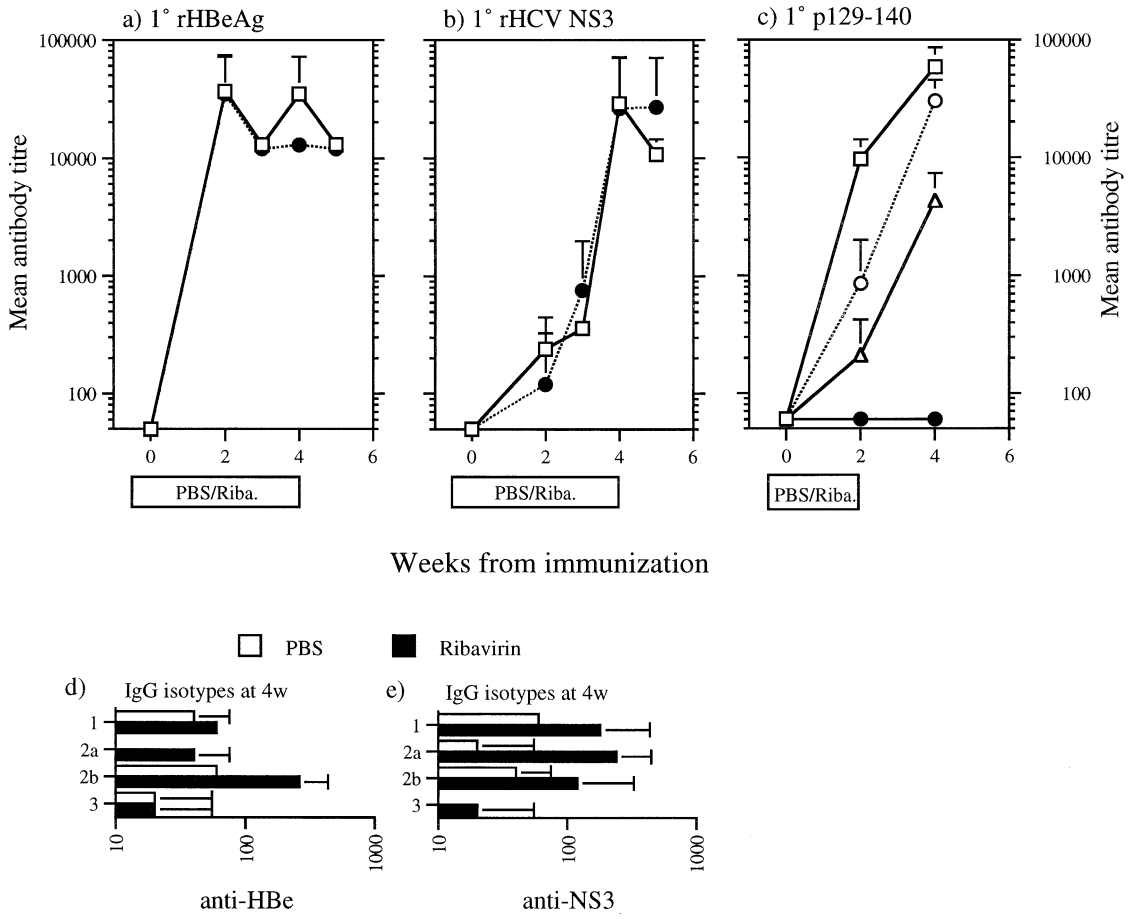

Fig. 4. *In vivo* effects of ribavirin treatment on the priming of polyclonal HBeAg- (*a*) and NS3- (*b*) specific humoral responses and on a Th2-mediated HBeAg-specific antibody response (*c*). Mice were bled on a weekly or bi-weekly basis and total anti-HBe IgG (*a*, *c*) or anti-HCV NS3 (*b*) titres were determined as described in Methods. Values are given as mean endpoint titres. (*a*)–(*b*) Groups of C57/BL6 (H-2^b) mice were primed with 50 μg rHBeAg (*a*) or 50 μg NS3 (*b*) in complete Freund's adjuvant at day 0, and were given PBS containing 0 (□) or 1·5 mg (●) ribavirin daily from days −3 to 28. Also shown are the IgG subclasses of anti-HBe (*d*) and anti-HCV NS3 (*e*) of the immunized mice in the week 4 samples. (*c*) Groups of HBeAg-Tg mice were primed with a synthetic HBc/eAg-derived T-cell site at day 0, and were given PBS containing 0 (□), 0·75 (○), 1·0 (△) or 1·5 (●) mg ribavirin daily from days −3 to 10.

treated mice at both week 4 (Fig. 4*c*; $R^2 = 0{\cdot}61$, $P < 0{\cdot}01$, ANOVA) and week 6 ($R^2 = 0{\cdot}71$, $P < 0{\cdot}01$, ANOVA). The highest dose of daily ribavirin completely prevented anti-HBe seroconversion whereas lower ribavirin doses reduced anti-HBe antibody titres (Fig. 4*c*).

To reveal the mechanism of inhibition of the anti-HBe seroconversion in the ribavirin-treated mice, the IgG isotypes of anti-HBe were determined in the week 4 samples. In agreement with previous observations (Milich *et al.*, 1995*b*, *c*), the control mice receiving PBS alone developed almost exclusively IgG1 anti-HBe antibodies (Fig. 5*a*). Similar to the total anti-HBe IgG response, the IgG1 anti-HBe levels were inversely proportional to the ribavirin dose ($R^2 = 0{\cdot}68$, $P < 0{\cdot}01$, ANOVA). In contrast, the highest IgG2a anti-HBe levels were found in the mice receiving the two lowest doses of ribavirin, and no linear relation was found between the IgG2a levels and the ribavirin dose (Fig. 5*a*). This indicates a differential effect of ribavirin treatment on the activated HBeAg-specific Th-cell phenotype *in vivo*.

To explain further the shift in anti-HBe IgG subclasses by ribavirin, groups of HBeAg-Tg mice were immunized s.c. with denatured HBeAg (which only primes T cells and does not have HBeAg antigenicity) and were treated for 9 days with daily injections of 100 μl PBS containing 0 or 1·0 mg ribavirin. *In vitro* recall of the primed T cells with HBeAg revealed comparable levels of IL-2, IL-4 and IL-6 in the PBS- and ribavirin-treated mice (Fig. 5*b–d*). In contrast, IFN-γ production could be recalled by levels of HBeAg as low as 0·008 μg/ml in the ribavirin-treated group whereas the PBS-treated group needed 625-fold higher levels (1 μg/ml) for detectable IFN-γ production (Fig. 5*e*). Thus, the dose-dependent decrease in anti-HBe IgG1 and increase in anti-HBe IgG2a are consistent with increased production of IFN-γ following ribavirin treatment.

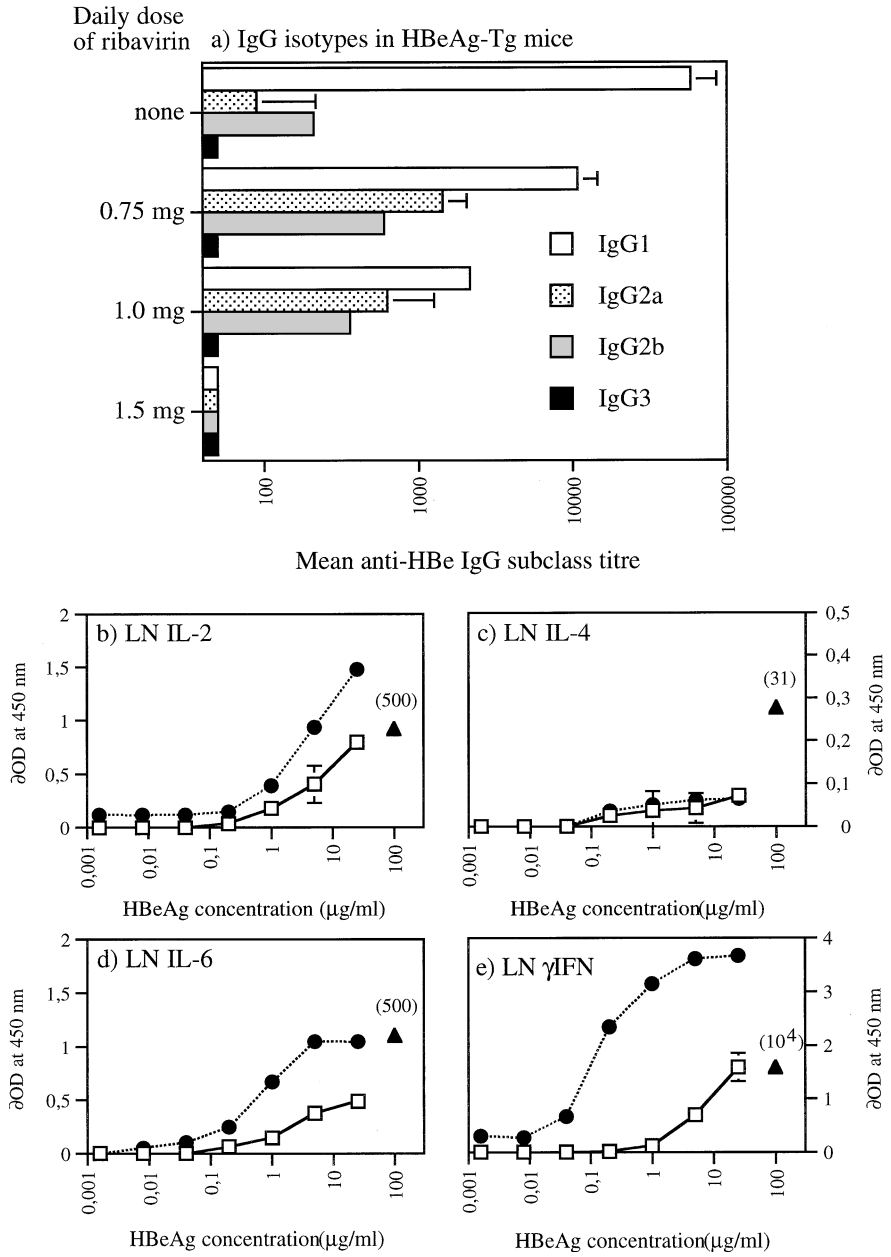

Fig. 5. (*a*) Anti-HBe IgG isotypes in HBeAg-Tg mice receiving PBS containing 0, 0·75, 1·0 or 1·5 mg ribavirin daily. Mice were bled every second week and titres of isotypes of anti-HBe IgG were determined as described in Methods. Values are given as the mean endpoint titres in samples taken 4 weeks after immunization. (*b*)–(*e*) *In vitro* recall by HBeAg of HBc/eAg-specific T cells from HBeAg-Tg mice given daily doses of PBS containing 0 (□) or 1·0 (●) mg ribavirin. Levels of cytokines determined in culture supernatants are shown. Values are given as the mean δOD at 450 nm. ▲, δOD obtained with the indicated amounts (in pg/ml, within parentheses) of the respective cytokines.

Discussion

There is *in vitro* evidence to illustrate the direct antiviral effect of ribavirin on a number of viruses. However, the *in vivo* effects of ribavirin on virus replication are not as clear. Ribavirin monotherapy appears to have no, or very limited, antiviral effect on HBV and HCV replication (Fried *et al.*, 1994; Kakumu *et al.*, 1993; Di Bisceglie *et al.*, 1995; Dusheiko *et al.*, 1996). However, in most patients there is a significant reduction in ALT levels during treatment which return to pre-treatment levels after stopping treatment (Di Bisceglie *et al.*, 1995; Dusheiko *et al.*, 1996; Reichard *et al.*, 1991). Ribavirin together with IFN-α seems to have beneficial effects in the treatment of chronic HCV infection (Lai *et al.*, 1996; Schvarcz *et al.*, 1995; Reichard *et al.*, 1998). We show that ribavirin treatment causes a transient drop in HCV-specific humoral

responses during treatment of patients with chronic HCV infection. Most importantly, the reduction was independent of virus load, suggesting a differential effect of ribavirin on the immune system. Cumulatively, these data suggest that the effects of ribavirin may be complex and not strictly antiviral.

Recent studies have indeed shown that ribavirin is immune-suppressive *in vitro*. Heagy *et al*. (1991) showed that ribavirin inhibits proliferation of polyclonally activated human PBMCs in a dose-dependent fashion. Ribavirin has also been shown to have inhibitory effects on cytokine production by RSV-infected epithelium (Mastronarde *et al*., 1996), and on the production of IL-6 in RSV-infected pulmonary epithelial cells (Jiang *et al*., 1998). Thus, similar effects may well be present *in vivo* during ribavirin treatment of chronic viral hepatitis.

It is possible that the anti-proliferative effect of ribavirin may be overestimated when measured as incorporation of [^{3}H]thymidine since ribavirin increases the cell pool of thymidine, thereby competitively reducing the radiolabelling of cellular DNA (Drach *et al*.,1980). However, microscopic inspection of polyclonally activated cell cultures treated with ribavirin shows that the reduction in the number of cells correlates positively with the amount of ribavirin added (unpublished observations). This highlights the importance of analysing several different parameters when evaluating the *in vitro* effects of ribavirin.

We have confirmed and extended previous findings on the inhibitory effect of ribavirin on T-cell activation *in vitro*. PHA-induced T-cell proliferation was inhibited at approximately 10-fold lower doses than was PHA-induced cytokine production. We found that *in vitro* IL-2 and IL-4 production were inhibited reproducibly at lower doses of ribavirin than was IFN-γ. Interestingly, similar observations were made when mice were treated with ribavirin during the priming of HBcAg-specific T cells. In both HBcAg- and HBeAg-immunized mice receiving ribavirin, *in vitro*-recalled IL-2 and IFN-γ production was detected at 25- to 125-fold lower HBc/eAg concentrations than in PBS-treated mice. These data suggest that ribavirin might act differentially on specific Th1- and Th2-like responses. It was recently shown that HBcAg and HBeAg differ in the Th cell phenotypes that they prime (Milich *et al*., 1997). This was also observed in the present study, since only HBcAg effectively primed IFN-γ-producing Th cells in the absence of ribavirin. Moreover, ribavirin did not seem to be directly immune-suppressive on B cells or on polyclonal responses promoted by a mixed Th1/Th2 subset. It is well-known that both Th1- and Th2-like cytokines promote humoral responses. Thus, a shift favouring Th1-like immune responses does not imply a reduction of humoral responses in a mixed Th1/Th2-like polyclonal immune response, since the reduced activity of one subset may be substituted for by activation of the other subset.

To test the effect *in vivo* of ribavirin treatment further, we studied how ribavirin affected an exclusively Th2-mediated HBeAg-specific immune response. Anti-HBe seroconversion in B10 $\times$ B10.S$_{F1}$ HBeAg-Tg mice can be induced by injection of an HBeAg-derived synthetic T-cell site (Milich *et al*., 1991). This seroconversion is mediated exclusively by HBeAg-specific Th2 cells, and the response can easily be modulated by compounds that affect either Th1- or Th2-like immune responses, such as IL-12 and IFN-α (Milich *et al*., 1995 *a*, *b*, *c*). High daily doses of ribavirin ($>$ 1 mg/day) applied to HBeAg-Tg mice totally inhibited anti-HBe seroconversion, clearly showing the immune-suppressive effects of ribavirin *in vivo*. This immune-suppressive effect may help to explain the normalization of ALT levels in patients with chronic HBV and HCV infection during treatment with ribavirin in the absence of a decrease in virus load (Di Bisceglie *et al*., 1995; Fried *et al*., 1994). Furthermore, an inverse relationship was found between ribavirin dose and the level of anti-HBe IgG1, an IgG isotype negatively regulated by Th1-derived cytokines (Stevens *et al*., 1988). In contrast, the highest levels of anti-HBe IgG2a, positively regulated by Th1-derived cytokines (Stevens *et al*., 1988), were found in two of the groups of mice receiving ribavirin. Thus, high doses of ribavirin ($>$ 1 mg/day) totally inhibited a primary and exclusively Th2-mediated antibody response but did not affect a primary antibody response containing a mixed Th1/Th2 HBc/eAg- or HCV NS3-specific T-cell phenotype. Lower doses of ribavirin suppressed the Th2-like HBeAg-specific response by causing a shift to a Th1-like immune response. We confirmed these observations on the T-cell level by *in vitro* recall of HBc/eAg-specific T cells from HBeAg-Tg mice, where IFN-γ production could be recalled at around 125-fold lower antigen concentrations in the ribavirin-treated group. Altogether this is in line with observations made by another group (R. C. Tam and others; unpublished observations).

The results of experiments on wild-type and HBeAg-Tg mice suggest that ribavirin exerts effects *in vivo*. The immune effects of ribavirin do not seem to be related to a particular antigen. Ribavirin does not seem to be directly immune-suppressive on B cells; instead changes in antibody levels or isotype distribution are more likely to be caused by effects on other factors, such as regulatory T cells. Finally, in ribavirin-treated HBeAg-Tg mice the decrease in anti-HBe IgG1 and increase in anti-HBe IgG2a are perfectly consistent with the observed increase in IFN-γ production by specific T cells.

Bearing in mind the concentration of the present study on murine responses, one may still speculate on the relevance of these findings to observations from ribavirin treatment of humans, especially in chronic viral hepatitis. First, normalization of ALT levels during ribavirin treatment of chronic HCV infection, despite no or only limited reduction in virus load, may be secondary to the immune-suppressive effects of ribavirin reported in this study. Second, the transient reduction of HCV antibodies during ribavirin treatment of humans, occurring independently of virus load, suggests the possibility of effects of ribavirin on humans similar to those indicated by the Th1/Th2 balance in HBeAg-Tg mice. Third,

the beneficial effect of treatment with ribavirin together with IFN-α, both of which compounds seem to favour Th1-like responses in mice, may be partly due to a shift in the Th1/Th2 balance (Milich *et al.*, 1997); whether this is also true of humans remains to be seen. However, since Th1-like immune responses are generally believed to have antiviral effects this shift may help to clear chronic HCV infection.

We observed recently that HCV NS3-specific Th cells become activated after cessation of treatment of chronic HCV infection with IFN-α alone, or in combination with ribavirin (Zhang *et al.*, 1997*b*). The observed delay in the development of HCV-specific proliferative T-cell responses might now be further explained by an immune-suppressive effect during the course of ribavirin treatment. We also noted that a change in the phenotype of NS3-specific T cells occurred after treatment. Prior to and during treatment NS3-specific T cells produced cytokines but did not proliferate, whereas they both produced IFN-γ and IL-10 and proliferated after treatment stopped (Zhang *et al.*, 1997*b*). This activation in humans is in line with the changes brought about by ribavirin during primary murine HBc/eAg-specific immune responses.

In summary, many of the previously noted effects exerted by ribavirin may be partly explained by a shift in the balance between Th1- and Th2-like immune responses *in vivo*. Thus, ribavirin may not be viewed strictly as an antiviral compound.

Financial support was obtained from the Swedish Cancer Foundation (grant no. 3825-B96-01XAB) and from The School of Dentistry at the Karolinska Institutet. Part of the present work was included in an oral presentation at 'Current Trends in Chronically Evolving Viral Hepatitis' on 11 October 1997, in Lyon, France.

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Received 2 June 1998; Accepted 2 July 1998