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Effects of IL-12 and IL-18 on HBcAg-specific cytokine production by CD4 T lymphocytes of children with chronic hepatitis B infection

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Abstract

The influence of IL-12 and IL-18 was evaluated on hepatitis B core antigen (HBcAg)-specific cytokine production (IFN-γ, IL-4, IL-5 and IL-10) by CD4 T lymphocytes isolated from peripheral blood of children with chronic hepatitis B. CD4 T cells were isolated from peripheral blood of 20 children with chronic active hepatitis B, cultured for 48 h in presence of rHBcAg and of co-stimulators, IL-12 or IL-18 or IL-12 + IL-18 or in their absence (control). Production of studied cytokines was examined using the ELISPOT assay. Co-stimulation with IL-12 or IL-18 was found to significantly augment the HBcAg-specific secretion of IFN-γ. However, the most pronounced stimulatory effect was observed in the presence of IL-12 + IL-18 and resulted in peak levels of IFN-γ production. The obtained results allowed concluding that the anti-HBV activity of Th1 lymphocytes is strongly induced by IL-12 + IL-18 and may contribute to viral clearance in children with chronic hepatitis B infection.

Keywords: Hepatitis B virus; Th lymphocytes; Interferon-γ; Interleukin-12; Interleukin-18

1. Introduction

Disturbed functions of CD4 T helper (Th) lymphocytes in the course of chronic hepatitis B have been well documented (Chisari and Ferrari, 1995; Löhr et al., 1995; Milich et al., 1997). In parallel, human CD4 T cells are known to be functionally heterogenous, containing two distinct Th subsets: Th1—producing interferon-gamma (IFN-γ) and IL-2, and Th2—releasing IL-4, IL-5 and IL-10 (Mosmann and Sad, 1996; O'Garra, 1998). In immunopathogenesis of chronic hepatitis B, a significant role seems to be played by a disturbed secretory function of Th1 cells, reflected by a weak or absent hepatitis B core antigen (HBcAg)-specific IFN-γ production (Milich et al., 1997; Jung et al., 1999; Koziel, 1999). Importance of IFN-γ in the antiviral T cell response to HBV has also been documented in the transgenic mouse model (Guidotti et al., 1994, 1999). IL-12, in turn, represents a recognized inducer of IFN-γ secretion by Th1 cells (Trinchieri, 1998; Trinchieri and Scott, 1999). It has also been demonstrated that IL-12 can inhibit the replication of hepatitis B virus (HBV) in transgenic mice through the induction of IFN-γ (Cavanaugh et al., 1997). IL-18 is known to act synergistically with IL-12 to enhance production of IFN-γ (Okamura et al., 1998).

The present study was aimed at the evaluation of IL-12 and IL-18 effects on HBcAg-specific cytokine production (IFN-γ, IL-4, IL-5 and IL-10) by Th lymphocytes in peripheral blood of children with chronic hepatitis B infection.

2. Materials and methods

2.1. Patients

The studies were conducted with a group of 20 children (14 boys and 6 girls), age 5–15 years (mean age of 8.5 ± 2.8 years), with well documented diagnosis of chronic hepatitis B. Duration of the HBV infection averaged at 2.6 ± 2.0 years. In none of the cases infection with type C virus co-existed. All children in the group showed the chronic hepatitis phase

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present for at least 6 months. In such children, serum ALT levels were augmented above 40 IU/l (mean value amounted to 78.6 ± 29.4 IU/l). In parallel, their sera manifested presence of HBsAg, HBeAg and HBV DNA (mean content of 64.4 ± 48.2 copies/ml × 10^6), while their liver biopsy material demonstrated histological signs of active chronic hepatitis. All the research protocols were reviewed and approved by the Ethics Committee by the Poznan University of Medical Sciences.

The control group consisted of 12 healthy children (8 boys and 4 girls), age 6–16 years (mean age of 8.1 ± 3.2 years) with no history of HBV infection. They were negative for HBV infection markers and showed normal serum ALT levels. Patients of the control group were studied as negative controls.

2.2. Serological markers of HBV and HCV infections

HBsAg, HBeAg, anti-HBs antibodies and anti-HBe antibodies were estimated in serum using commercial ELISA tests (Microwell system, Organon Teknika). Serum anti-HCV antibodies were estimated using UBI HCV EIA tests (Organon Teknika). Serum HBV DNA was quantitated using hybridization and the hybrid capture system with chemiluminescence readout (HBV DNA Assay, Digene), employing Pharmacia luminometer.

2.3. Isolation of CD4 T lymphocytes from peripheral blood

Peripheral blood mononuclear cells (PBMC, 80–90% lymphocytes + monocytes) were isolated from heparinised blood by centrifugation in the Ficoll/Isopaque density gradient (spec. density 1.078 g/cm^3, Pharmacia) for 20 min at 400 g. The interface cells were washed twice in PBS, scored and suspended in PBS, enriched with 2% fetal calf serum (FCS; Gibco), at 10^7 PBMC/ml. CD4 T lymphocytes were isolated by the biomagnetic technique using Dynabeads M-450 CD4 (Dynal) (Funderud et al., 1987). The obtained cell preparation contained 90–95% CD4 T cells, as demonstrated by direct immunofluorescence using phycoerythrin-labelled monoclonal anti-CD4 antibodies (CD4/RPE; Dako) and 5–10% monocytes. Cell viability was tested using 0.5% trypan blue (following every isolation the percentage of dead cells did not exceed 5%). The isolated cells were washed twice and suspended to 10^6/ml RPMI 1640 (Sigma), enriched with 10% FCS, 2mM L-glutamine (Gibco), 50 μg/ml of gentamycin and 200 IU/ml of penicillin.

2.4. ELISPOT assay

Detection of IFN-γ, IL-4, IL-5 and IL-10 was performed using ELISPOT kits (Mabtech). Cultures of the isolated cells (CD4 T lymphocytes/monocytes; 10^5 per well) were established on Multiscreen 96-well plates (Millipore) coated with appropriate monoclonal anti-IFN-γ, anti-IL-4, anti-IL-5 or anti-IL-10 antibodies, according to the manufacturer's instructions. Cell cultures were conducted in presence of 1 μg/ml recombinant HBcAg (rHBcAg, 90% pure, as estimated by Coomassie blue stained SDS-PAGE; Intracel) and of co-stimulators: 1 ng/ml IL-12 (R&D) or 10 ng/ml rIL-18 (MBL) or 1 ng/ml IL-12 + 10 ng/ml IL-18 for 48 h at 37 °C (5% CO_2). The applied concentrations of IL-12 and IL-18 in preliminary experiments were found to maximally induce HBcAg-specific secretion of IFN-γ in cultures of CD4 T lymphocytes isolated from the patients. In parallel, control cultures (of patients and healthy children) were established with HBcAg alone or without HBcAg or without co-stimulators (IL-12 and IL-18) as well as control cultures stimulated with IL-12 or IL-18 alone. Cytokine spots were visualised by biotin-labelled antibodies (ELISPOT for cytokine detection; Mabtech) (El Ghazali et al., 1993). Number of cytokine spots was established using Nikon Eclipse E600 microscope and LeicaQ500MC computer software (Leica). Antigen-specific stimulation of studied cytokine release was evaluated by the stimulation index (SI), expressing ratio of spots obtained in presence of antigen with IL-12 and/or IL-18 and those obtained with antigen alone.

2.5. Statistical analysis

The results are expressed as the mean ± standard deviations (S.D.). The data were analyzed using paired *t*-tests. The *P*-values of <0.05 were regarded as significant.

3. Results

Secretion of tested cytokines (IFN-γ, IL-4, IL-5 and IL-10) by peripheral blood CD4 T lymphocytes of children with chronic hepatitis B in non-stimulated cultures and in presence of IL-12 and/or IL-18 (controls) are presented in Fig. 1A. Production of the cytokines by non-stimulated lymphocytes was very low: mean values for IFN-γ, IL-4, IL-5 and IL-10 amounted to, respectively, 1.65 ± 1.5, 1.95 ± 1.1, 1.95 ± 1.2 and 4.45 ± 0.82. In cultures with IL-12 or IL-18, mean number of IFN-γ spots was significantly augmented, as compared to cultures with medium alone (mean number of IFN-γ spots in IL-12-containing cultures: 3.55 ± 1.28, in IL-18-containing cultures: 2.85 ± 0.93) although secretion of the remaining cytokines (IL-4, IL-5 and IL-10) showed no significant changes. In cultures with IL-12 and IL-18 in parallel also mean number of IFN-γ was significantly augmented (to 4.9 ± 0.91), as compared to cultures with medium alone, while mean values for IL-4, IL-5 and IL-10 spots showed no significant alterations.

Secretion of tested cytokines (IFN-γ, IL-4, IL-5 and IL-10) in 48-h cultures of CD4 T lymphocytes, obtained from children with chronic hepatitis B, in presence of HBcAg alone or in presence of the antigen plus IL-12 and/or IL-18 are shown in Fig. 1B. In response to HBcAg alone, secretion of IFN-γ (mean number of IFN-γ spots: 3.9 ± 1.46),

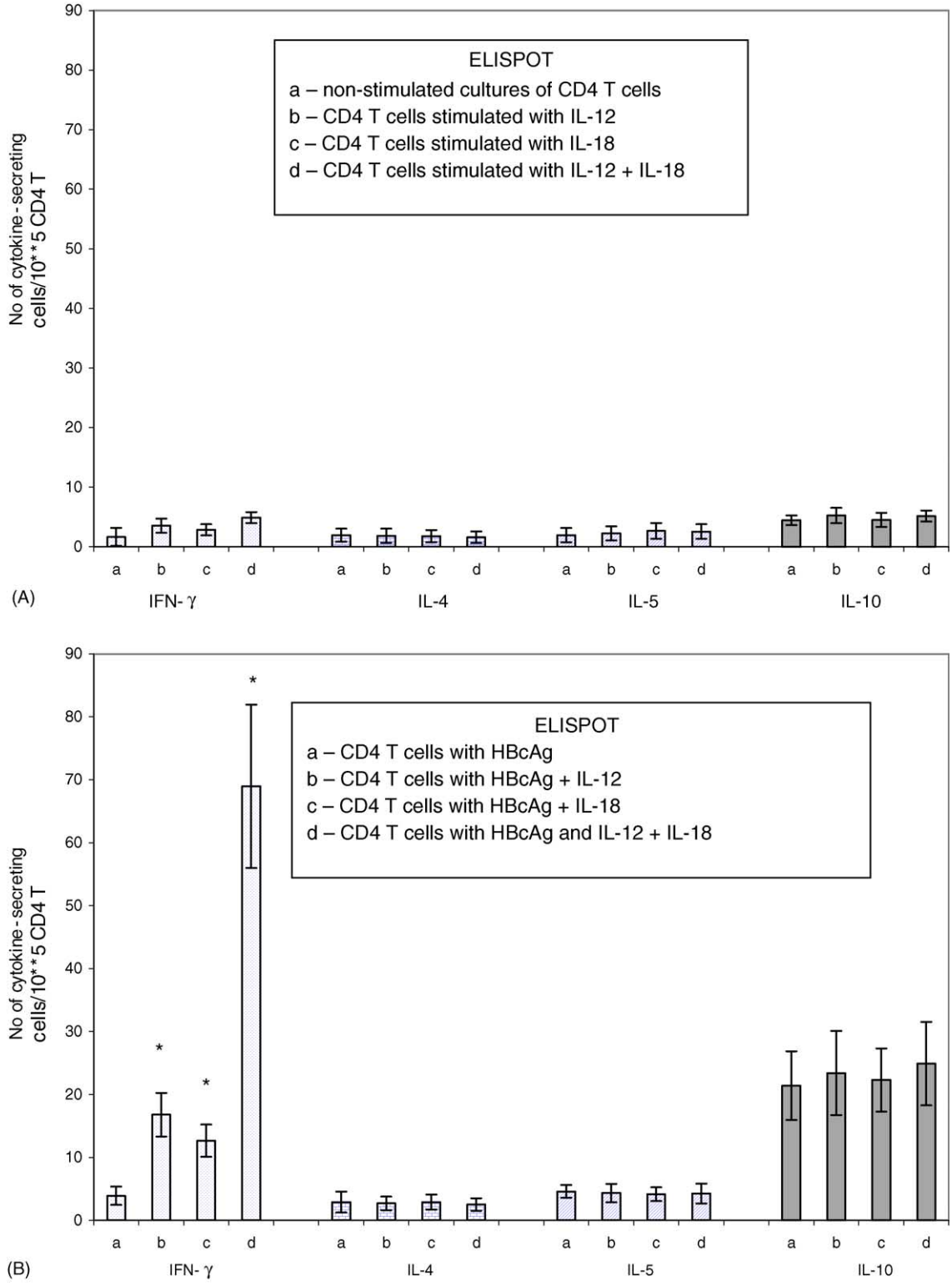

Fig. 1. (A) Production of cytokines by peripheral blood CD4 T lymphocytes of children with chronic hepatitis B in studied cultures. (B) Influence of IL-12 and/or IL-18 on HBcAg-specific cytokine production by peripheral blood CD4 T lymphocytes in children with chronic hepatitis B (means ± S.D. bars). (*) Significanthy different from cultures not co-stimulated with IL-12 and/or IL-18 ($P < 0.05$).

IL-4 (mean number of IL-4 spots: 2.9 ± 1.64), IL-5 (mean number of IL-5 spots: 4.6 ± 1.02) was low and significantly lower than secretion of IL-10 (mean number of IL-10 spots: 21.4 ± 5.45). In cultures with HBcAg and IL-12, secretion of IFN-γ (mean number of IFN-γ spots: 16.8 ± 3.46) was significantly higher than secretion of the cytokine in response to HBcAg in the absence of IL-12. On the other hand, release of the remaining cytokines, IL-4 (mean number of IL-4 spots: 2.7 ± 1.08), IL-5 (mean number of IL-5 spots: 4.35 ± 1.46) and IL-10 (mean number of IL-10 spots: 23.4 ± 6.68) did not significantly differ from production of the cytokines in the absence of IL-12. In cultures with HBcAg and IL-18, secretion of IFN-γ (mean number of IFN-γ spots: 12.65 ± 2.56) was significantly higher than production of the cytokine in response to HBcAg in absence of IL-18. On the other hand, release of the remaining cytokines, i.e., of IL-4 (mean number of IL-4 spots: 2.9 ± 1.2), IL-5 (mean number of IL-5 spots: 4.15 ± 1.09) and IL-10 (mean number of IL-10 spots: 22.3 ± 5.0) did not significantly differ from production of the cytokines in absence of IL-18. In the cultures with HBcAg and IL-12 + IL-18, secretion of IFN-γ (mean number of IFN-γ spots: 68.95 ± 12.97) was significantly higher as compared to production of the cytokines in response to HBcAg in absence of IL-12 + IL-18. On the other hand, release of the remaining cytokines, IL-4 (mean number of IL-4 spots: 2.5 ± 1.0), IL-5 (mean number of IL-5 spots: 4.25 ± 1.59) and IL-10 (mean number of IL-10 spots: 24.9 ± 6.62) did not significantly differ from production of the cytokines in absence of IL-12 + IL-18.

4. Discussion

In the performed studies on antigen-specific secretory response of CD4 T lymphocytes, ELISPOT assay has been employed, allowing to assess the frequency of cytokine-producing cells and the kind of lymphokine produced (Jung et al., 1999). High sensitivity of the assay has allowed to use it for analysis of quantitative changes in cytokine-secreting cells in cultures of lymphocytes T in presence of IL-12 and/or IL-18. Also, the obtained results of stimulation indexes in cultures with HBcAg co-stimulated with IL-12 and/or IL-18 have confirmed antigen specificity of the augmentation of cytokine production.

The cytokine secretory response of CD4 T lymphocytes originating from peripheral blood of chronic hepatitis B patients, when stimulated with HBcAg in vitro, demonstrated an inhibited function of Th1, as reflected by a reduced release of IFN-γ, and of Th2 cells, as reflected by a deficient release of IL-4 and IL-5 [consistent with an earlier report (Szkaradkiewicz et al., 2003)]. In parallel, the results documented preferential HBcAg-specific production of IL-10. In view of the presented data, it seems probable that the demonstrated secretion of IL-10 has reflected antigen-specific activation of monocytes present in the tested cultures and/or a specific induction of recently described regulatory CD4 T lymphocytes (T_R) (Moore et al., 1993; Maloy and Powrie, 2001). At present, antigen-stimulated clones of T_R are thought to suppress immune response via the release of the cytokines IL-10 and transforming growth factor-β (TGF-β) (Yazdanbakhsh et al., 2002). In chronic active B hepatitis in children, the preferentially produced IL-10 may significantly down-regulate the immuno-inflammatory attack on liver cells, resulting in diminished progression of the liver damage.

In studies on HBcAg-specific cytokine production in IL-12-containing cultures of CD4 T lymphocytes from peripheral blood of children with chronic hepatitis B increased IFN-γ secretion was noted, while secretion of the remaining tested cytokines was not significantly different from control cultures. The data are consistent with earlier presented results obtained in patients with chronic hepatitis B, expressing serological markers of active chronic HBV infection (HBsAg, HBeAg and HBV-DNA) (Schlaak et al., 1999). In turn, our results have failed to confirm the suggestion that the regulatory effect of IL-12 on T cell response is mediated through IL-10 production (Schlaak et al., 1999; Meyaard et al., 1996). Possibly, IL-12 exerts no in vitro effect on in vivo HBV-activated clones of lymphocytes T that are releasing IL-10.

Examining HBcAg-specific production of cytokines in IL-18-containing cultures of CD4 T cells from peripheral blood of children with chronic hepatitis B, a weak but significant increase in the release of IFN-γ has been noted, while secretion of the remaining cytokines was not significantly different from the control cultures. The biological activity of IL-18 has already been well documented by demonstration that this cytokine acts as a co-stimulatory factor on Th1 cells (Kohno et al., 1997). Our data are consistent with those reported.

Analysis of HBcAg-specific cytokine production in cultures of CD4 T lymphocytes, isolated from the peripheral blood of children with chronic HBV revealed that the most pronounced stimulatory effect was obtained in the presence of IL-12 + IL-18 and involved the secretion of IFN-γ. The IFN-γ secretion obtained with IL-12 and IL-18 confirmed the synergistic action of the two cytokines on the induction of IFN-γ synthesis in Th1 cells, as previously demonstrated by other (Okamura et al., 1998; Hunter and Reiner, 2000). In contrast to the strong stimulatory effect of IL-12 + IL-18 on HBcAg-specific IFN-γ production by Th1 lymphocytes, no antigen-specific secretory response of "non-Th1" cells was observed. The lymphocyte Th2-typical and HBcAg-specific defect in release of IL-4 and IL-5 remained unchanged following co-stimulation with IL-12 and IL-18, similarly to the unchanged high IL-10 production.

Thus, the present results indicate that synergistic action of IL-12 and IL-18 results in a strong activation of HBcAg-specific Th1 lymphocytes, witnessed by a high IFN-γ production. In turn, the IL-12- and IL-18-induced stimulatory effect on Th1 lymphocytes was not accompanied by suppressive effects on the HBcAg-specific activation of most probably "non-Th" cells, which release IL-10. The latter effect may decrease efficiency of the antiviral response. Nevertheless,

the anti-HBV activity of Th1 lymphocytes, strongly induced by IL-12 and IL-18 and reflected by a high secretion of IFN-γ, markedly surpassing IL-10 production, might be sufficient for viral clearance in children with chronic hepatitis B infection. Possible therapeutic potential of IL-12 and IL-18 application in chronic hepatitis B requires further studies.

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