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The flavonoid ellagic acid from a medicinal herb inhibits host immune tolerance induced by the hepatitis B virus-e antigen

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Abstract

The aim of this study is to characterize the role of ellagic acid, a flavonoid from a medicinal herb which blocks HBeAg secretion in a HBV infected cell line and in HBeAg transgenic mice, in immune tolerance in chronic HBV infection. Using the mouse strain C57ML/6, HBeAg-producing transgenic mice (HBeAg-Tg), under the control of metal ion-inducible promoter were generated. The effect on immune tolerance of HBeAg-Tg and the release of immune tolerance by the inhibitor of HBeAg secretion, ellagic acid, was tested using T/B cell proliferation, HBeAg/HBeAb production, cytotoxic T-lymphocyte (CTL) and cytokine assays.

C57ML/6 based HBeAg-producing HBeAg-Tg mice were tolerant to HBeAg at the T and B-cell level, did not produce antibodies to HBeAg in vivo and in vitro, produced minimal levels of cytokines (IL-4 and IFN-gamma) and decreased CTL responses, while feeding mice with ellagic acid (5 mg/kg body weight) blocked the immune tolerance caused by HBeAg. Our results suggest that host immune tolerance induced by HBeAg during HBV infection, a viral strategy to guarantee HBV infection, can be overcome by ellagic acid, thus it can be used as a therapeutic for HBV-carriers.

Keywords: Hepatitis B virus–e antigen; Immune tolerance; Ellagic acid; Anti-HBV infection

1. Introduction

The human hepatitis B virus (HBV) causes acute and chronic hepatitis and is closely related to the high incidence of cirrhosis and hepatocellular carcinoma. Among the four HBV encoded viral proteins, the HBV-e antigen (HBeAg), suspected to promote chronicity of the viral disease, is encoded by the core gene (Ganem et al., 1987). HBeAg is the secretory nonparticulate form of HBcAg, which is not required for viral replication or infection. Many studies suggested that HBeAg modulate the complex interaction between HBV and the immune system, and thereby promotes the chronicity of the viral disease (Chen et al., 2004; Millich et al., 1990). The HBV is not directly cytopathic and the immune response of the host appears to mediate hepatocellular and tissue injury and subsequent viral clearance (Orito and Mizokami, 2005). The vast majority of untreated infants born from HBeAg-positive chronic carrier mothers become infected and more than 90% of them become chronic carriers (Holtby and Macarron, 2004). In contrast, in individuals who were born in HBeAg(-) mother, more than 90% of HBV infections occurring in adults are resolved as acute infections, and only 5–10% result in chronic infection (Aldershvile et al., 1980). This dramatic difference in chronicity rates is believed to reflect the immunologic status of the host at the time of infection. Neonates born to HBV carrier mothers are immunologically tolerant to viral proteins to which they were exposed in utero (Thomas et al., 1998). Studies into the role of immunologic tolerance in chronic infection of the newborn, using the HBeAg-expressing transgenic mice model suggested that HBeAg can cross the placenta and establish T-helper (Th) cell tolerance in utero specific for HBcAg and HBeAg (Millich et al., 1990). Therefore, it was confirmed that HBeAg induces immunologic tolerance in utero and is responsible for the high chronicity rates (∼90%) observed in babies infected prenatally by their

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HBe-Ag-positive mothers. Expression of HBeAg maybe one of the viral strategies aimed to guarantee persistence during vertical transmission of HBV, which is the major source of chronic infection in areas where HBV is endemic. It is known that a variety of naturally occurring substances have the ability to protect against certain types of human diseases. Plant phenols are one important category of such naturally occurring chemopreventive agents (Priyadarsini et al., 2002). Ellagic acid is a polyphenol that occurs largely as ellagitannins in woody dicotyledon plants, such as grapes, strawberries, blacks currants and raspberries and showed variety of biological activities including antioxidant, anti-inflammatory and anti-fibrosis activities. Recently, we reported on the inhibitory effect of ellagic acid, isolated from *Phyllanthus urinaria*, on HBeAg secretion in HepG2 2.2.15 cell line (Shin et al., 2005). In the current study, we have tested the effect of ellagic acid on the relief of immune tolerance of the HBeAg-producing transgenic animals. Results from T and B cell proliferation, HBeAg/HBeAb production, CTL and cytokine detection assays suggested that ellagic acid, a HBeAg secretion blocker, is involved in the relief of immune tolerance, caused by HBeAg in HBeAg producing transgenic mice.

2. Materials and methods

2.1. Serology

The HBeAg level was measured in diluted transgenic mouse sera or cell culture supernatants with a commercial ELISA kit (Enzygost HBe monoclonal EIA kit, Dade Behring, Marburg, Germany). Anti-HBe antibodies, in mouse sera or in cell culture supernatants were measured by indirect solid-phase ELISA using HBeAg (100 ng per well) coated plates (Enzygost HBeAb ELISA kit). Briefly, serially diluted sera or supernatants were distributed into the HBeAg coated wells, and incubated at 37 °C for 4 h. After incubation for 1 h with 100 μl peroxidase conjugated anti-mouse IgG, the TMB substrate was added and OD was measured.

2.2. Production of transgenic mice

The transgenic mouse lineage from C57ML/6, a H-2^b genotype background with black color, was allocated from NIH, USA and maintained by KRIBB animal center. HBeAg-producing Tag mouse was generated at KRIBB animal centre by standard procedure (12). Briefly, the viral genomic DNA fragment (Subtype ad, coordinates 1804–2804) containing the complete procure plus core open reading frame (ORF) was PCR amplified and cloned between the mouse metallothionein I promoter (MTp) and polyadenylation recognition sequences of pMT-SV40 vector. The resulting expression vector, pMT-SV40-eAg, was digested with *Xho* I and microinjected into the male pronucleus of fertilized one cell ova of C57ML/6 mice. The progeny were screened for the presence of the microinjected DNA by PCR amplification of DNA isolated from mouse tail by using two sets of primers that would yielded products of 1017 bp and 659 bp, respectively (external primers: primer 1 – aaa/agc/ttc/acc/atg/aaa/cct/ttt/tca/ec, primer 2 – aag/aat/tca/atg/aag/cgc/tgc/gtg/tag/t and internal primers: primer 3- aaa/agc/ttc/acc/atg/aaa/cct/ttt/cac/c, primer 4 – aag/aat/ttc/taa/cat/tgg/gat/tcc/cga/g) under the following conditions; 94 °C for 1 min, 58.3 °C for 1 min, 72 °C for 1 min, for 35 cycles and a final extension at 72 °C for 5 min. For nested PCR, 1 μl of the reaction mixture was used in subsequent PCR with the internal primers using, 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min for 35 cycles and final extension ant 72 °C for 5 min. The inbred transgenic mouse C57 ML/6 lineage was derived by breading the founder mouse with transgenic mice.

2.3. Isolation of ellagic acid

Ellagic acid was isolated and purified from the medicinal plant, *Phyllanthus urinaria* as described (11).

2.4. Feeding of ellagic acid

Each group of normal C57ML/6 (control), C57ML/6-HBeAg-Tg (Tg) and ellagic acid (5 mg/kg body weight) fed Tg mice were maintained in the animal laboratory of KRIBB during the entire period of the experiment. Normal and Tg mice were supplied with double-distilled water for drinking whereas the other group of mice received ellagic acid (5 mg/kg body weight) dissolve in double distilled water. Ellagic acid in drinking water was prepared fresh on every Monday, Wednesday and Friday so that each mice (approximately 40 g body weight) was able to take 200 μg ellagic acid per day. These doses of ellagic acid were well tolerated by the animals with no apparent sign of toxicity such as weight loss or mortality.

2.5. T and B cell proliferation assay

Three groups of mice (Contol, Tg and Tg + EA), at least five mice for each group, were primed with either 2 μg of HBeAg emulsified in complete freunds adjuvant (CFA) at pH 7.2 or 9.6 by hind footpad injection. Ten days after immunization, the spleen cells were harvested from individual mice and T and B cells were isolated using Ficoll/Plaque plus solution (Amersham Bioscience, Uppsala, Sweden). Approximately, 1×10^6/ml of T and B cells were plated in 96-well plates in triplicate. After 48 h incubation with increasing concentrations of HBeAg (0.001, 0.01, 0.1 and 1.0 μg), 20 μl of MTT (3-4.5-dimethylthiazeol-2-yl)-2.5-diphenyl tetrazolium bromide (Sigma, MO, USA) was added to each well and incubated for 4–5 h. Solubilization solution (0.01 N, 10% SDS) was then added to each well. After vigorous mixing of the solution with a multichannel pipette, the plate was left overnight at 37 °C and the absorbance were read in a multichannel reader at 570 nm.

2.6. In vivo HBeAg and anti-HBeAb production assays

To examine the HBeAg and anti-HBeAb status in vivo, groups of five mice were primed intradermally with 2.0 μg

of HBeAg emulsified in CFA. Two weeks later, 2 μg HBeAg was given to each mouse. Sera were collected 3, 4, 5 and 6 weeks after the boost immunization and were analyzed for HBeAg and anti-HBeAb by solid-phases ELISA as described. The HBeAg titer was expressed as U/ml while the HBeAb titer was expressed as the reciprocal of the dilution (1/log $_4$) of serum.

2.7. In vitro anti-HBe production

To examine anti-HBe antibody production groups of five mice were primed intradermally with 2.0 μg of HBeAg emulsified in CFA. Two weeks later 2 μg of HBeAg boost was given to each mouse. Ten days later, the spleen cells (3.5×10^6 cells per ml) taken from the immunized animals, were cultured in RPM 1640 medium containing 5% fetal calf serum with or without HBeAg (2 μg/ml). Cell culture supernatants were harvested on day 4 and analyzed for IgM, IgA, IgG1, IgG2a, IgG2b or IgG3 anti-HBe antibodies by indirect solid-phase ELISA using IgM, IgA and IgG subclass- specific secondary antibodies (Pierce, Rockford., IL).

2.8. Cytotoxic T-lymphocyte assay

For the preparation of effector cells, three groups of mice were primed with 2 μg of HBeAg, boosted 2 weeks later, with 2 μg of HBeAg and sacrificed in 2 weeks. T and B cells isolated cells from spleens were prepared in the RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to the CTL assay, a target cell line, H-2D^b mouse fibroblast EL4, established from a lymphoma induced in C57BL mouse by 9, 10,-dimethyl-1, 2-benzanthracene, was transiently transfected with pMT-SV40-eAg plasmid and maintained in RPMI 1640 (GibcoBRL, NY USA) supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. After 24 h, the target cells were inactivated by mitomycin C treatment (25 μg/ml) overnight. For the CTL assay, viable T-cells were incubated with various numbers of EL4 cells in microtiter plates for 4 h. For the control, empty vector (pMT-SV40) trsnfected EL4 was incubated with lymphocytes from normal mice. The cytolytic activity of in vitro stimulated CTL was measured by LDH assay (Takatra, Shiba, Japan). The LDH reaction mixture was added to the collected 100 μl of cell supernatants and incubated for 30 min at room temperature before OD was measured at 490–492 nm.

2.9. Cytokine assay

Control, HBeAg-Tg and EA fed HBeAg-Tg mice were immunized with HBeAg. Two weeks later, they were boosted with 2 μg of HBeAg and sacrificed after 10 days. Then, lymphocytes were isolated from spleens and cultured in RPMI 1640 media. After 4 and 7 days in culture, supernatants were collected and analyzed for the presence of IFN-γ and IL-4 using ELISA kits for mouse IFN-γ and IL-4 (Assay Designs, IL-USA).

3. Results

3.1. Generation of HBeAg producing transgenic mice

The DNA fragment (subtype adr, coordinates 1804–2804) containing the complete precore plus core ORF (Fig. 1A) was PCR amplified and cloned into the T-vector which has the poly T sequences in both 5′ and 3′ ends of the vector (Fig. 1B). *EcoR* I/*Hind* III DNA fragment from the T-preC-C construct was subcloned into the pMT-SV40 between the MTp and polyA sequences so that the expression of HBeAg was controlled by the MTp (Fig. 1B). The transgenic mice were screened for the presence of the microinjected DNA by PCR amplification (Fig. 1C). Expression of the transgene would result in the synthesis and secretion of HBeAg, into the serum of the mice. Analysis of transgenic mouse serum by ELISA demonstrated that HBeAg was present and the level increased 3–4-fold by Zn^{++} administrations (Fig. 1D).

3.2. Ellagic acid induced a T/B cell immune response in immunologically tolerant HBeAg-transgenic mice

To determine the T/B cell response, at least five mice in each three groups, control, HBeAg- Tg or EA fed HBeAg-Tg mice were immunized with HBeAg and T/B cell proliferation assay specific for HBeAg was performed (Fig. 2). HBeAg-primed T/B cells of control mice responded strongly to HBeAg while HBeAg-primed T/B cells of Tg mice were immune-tolerant to HBeAg. Therefore, HBeAg-expressing Tg mice are functionally immune-tolerant to HBeAg at the T/B cell level. The T/B-cell tolerance did not appear to involve T suppressor cells as HBeAg-primed T/B cells from transgenic mice did not inhibit the proliferation of HBeAg-primed T/B cells from control mice (data not shown). In order to test the effect of ellagic acid on HBeAg-primed transgenic mice, ellagic acid (5 mg/kg body weight) were fed to transgenic mice for 6 weeks and the lymphocyte proliferation assay was performed. As shown in Fig. 2, ellagic acid fed HBeAg primed Tg mice showed T/B cell response much stronger than that of HBeAg primed Tg mice and more than 60% to that of the control mice. The effect of ellagic acid on the T/B cell proliferation was specific to the immune tolerance by HBeAg, since the effect of increased concentrations of in vitro HBeAg on the isolated lymphocytes, which appeared as MTT assay results, were proportional to the T/B cell proliferations.

3.3. In vivo antigen, antibody production in HBeAg-Tg and EA fed HBeAg-Tg mice

To test the effect of EA on the production of HBeAg and HBeAb, sera were collected from Tg mice that had been immunized and boosted as described in Materials and Methods. In HBeAg-Tg mice, 1.1–1.2 U/ml HBeAg(equivalent to 11–12 ng/ml) were present in the sera.

As showed in Fig. 3A, the HBeAg levels in sera in Tg mice remained constant while the levels decreased in EA fed Tg mice. In control mice, a background level of HBeAg was detected in the serum.

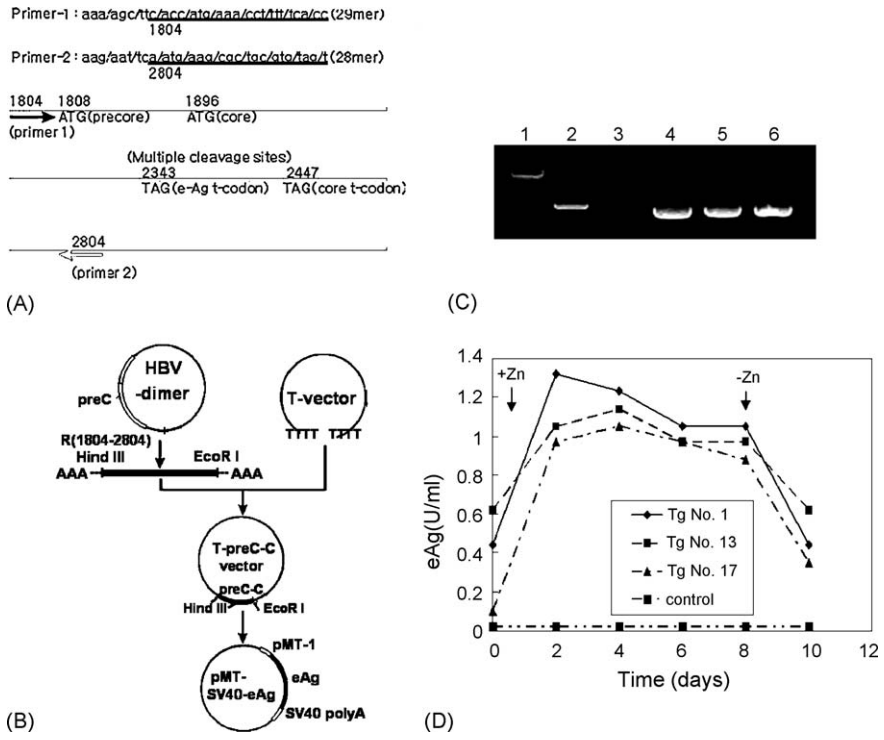

Fig. 1. HBeAg expression in transgenic mice. (A) PCR amplification of a DNA fragment containing pre-C, core region (nt 1804 ~ nt 2804) of the HBV genome, using primer 1 (29 mer) and primer 2 (28 mer), as described in the materials and methods. (B) Construction of the HBeAg expression vector, under pMT-1 (mouse metallothionein I promoter) control for microinjection into mice. (C) Southern blot analysis of the HBeAg transgenic mouse genome. Mouse genomic DNA from the tail was digested with BamH1, and probed with primer 1 and 2 for the PCR detection of preC-C ORF of HBV DNA fragment (coordinated 1804–2804). (d) Induction of serum HBeAg in HBeAg-Tg mice. Induction of the MT promoter was performed by administration of 25 mM zinc sulfate in the drinking water (+Zn). Subsequently, the mice were given normal dinking water (−Zn). The HBeAg-Tg mice which showed lower HBeAg basal level than 0.2 U/ml, were used.

HBeAg also showed complete nonresponces after primary and secondary boosting of HBeAg as shown in Fig. 3B. HBeAg-Tg did not produce anti-HBeAb after boosting with antigen.

Our results coincide with Milich et al's results (3) on B10.S-Tg31 mice which showed complete nonresponsiveness after primary immunization and produce only minimal anti-HBeAb after second immunization, though F1-Tg31e mice(which were generated by B10.S X B10.s-Tg31) produced comparable levels of anti-HBeAb. F1-Tg31e mice are significantly less tolerant than B10.S-Tg31e mice to the Tg self HBeAg in antibody productions and elicited anti-HBeAb productions sufficient to neutralize the detection of HBeAb. In Fig. 3B, the EA fed HBeAg-Tg mice started to produce anti-HBeAb, 6 days after the HBeAg boost. Since the HBeAg-Tg mice did not produce anti-HBe antibodies, this result suggest that HBeAg-specific B cells are tolerant in these mice while EA feeding helped HBeAg-specific B-cells become intolerant. Therefore, the enhanced anti-HBeAb production in EA fed HBe-Tg mice reflect a significant recovery of Th-cell function in EA fed HBeAg-Tg mice.

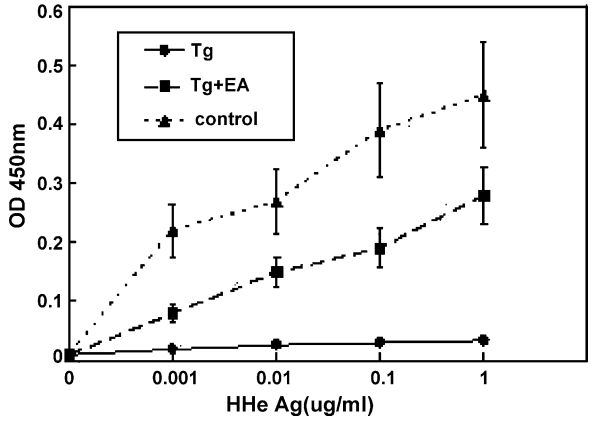

Fig. 2. T/B cell response of control (C57ML/6), HBeAg-Tg mice and EA-fed HBeAg-Tg mice upon immunization with HBeAg. Five mice of the control, HBeAg-Tg and EA fed HBeAg-Tg were immunized with 2 μg of HBeAg, and draining spleen cells were harvested 10 days later. The T/B cell proliferation response, induced by incubation for 48 h with various concentrations of the indicated antigens was determined. Each data point represents the mean (±S.D.) OD values obtained from 5 mice.

3.4. *In vitro anti-HBeAb production in HBeAg-Tg and EA fed HBeAg-Tg mice*

To test the HBe-specific Th-cell function directly in HBeAg-Tg mice and EA fed Tg mice, in vitro anti-HBeAb production was determined. As shown in Fig. 4, HBeAg primed and boosted spleen cells of normal mice produced IgM and four subtypes of IgG anti-HBeAbs after 4 days in culture. In contrast, HBeAg primed HBe-Tg mice spleen cells produced only IgM and IgA (T-cell independent) anti-HBeAb after 4 days in culture. The HBeAg-Tg pattern of in vitro anti-HBe production and reduced IgG subtype anti-HBeAb production in vitro indicated a HBeAg-

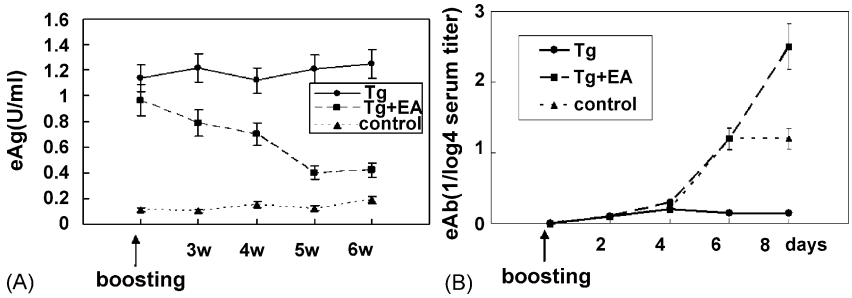

Fig. 3. In vivo antigen, antibody production in control C57ML/6, HBeAg-Tg and EA-fed Tg mice. Five mice of control, HBeAg-Tg or EA fed HBeAg-Tg mice were immunized with 2 μg of the HBeAg and boosted with 2 μg HBeAg in 2 weeks. Sera were collected 3, 4, 5 and 6 weeks after boosting and were analyzed for HBeAg(A), and anti-HBeAb(B) by solid-phase ELISA. The antigen titer was expressed as units/ml while the antibody titer was expressed as the reciprocal of the dilution ($1/\log_4$) of serum.

specific Th-cell deficit compared with control C57ML/6 mice. The lymphocytes isolated from EA fed HBe-Tg mice were also tested for the HBe-specific Th-cell function. As shown in Fig. 4, in contrast to HBeAg-Tg mice, EA fed Tg mice spleen cells produced IgG type anti-HBeAbs as well as IgM and IgA HBeAbs after 4 days in culture. The recovered antibody production in EA fed HBeAg-Tg indicated that the HBeAg-specific Th-cell deficit became relived. EA feeding on HBeAg-Tg mice activated HBeAg derived Th cells to produce sufficient anti-HBe "antoantibody" to complex with and reduce detection of serum HBeAg. These observations suggested the possibility that the EA affected on T-cell tolerance in HBeAg-expressing Tg mice extended to activation of Th cells as well as to proliferating T-cells.

3.5. Increased cytotoxic T-cell responses in EA fed HBeAg transgenic mice

To examine whether HBeAg-specific CTL in control, HBeAg-Tg and EA fed HBeAg-Tg mice, mouse was immunized with 2 μg HBeAg and boosted 2 weeks later. Spleen cells were isolated and incubated with target cells (pMT-SV40-eAg transfected EL4) at various effector/target cell ratios (100/1, 50/1, 25/1 and 12.5/1) for 5 h and the LDH assay was performed with 100 μl of supernatant (Fig. 5). These result suggested that HBeAg-induced tolerance in CTL response was attenuated by EA feeding.

3.6. EA induced cytokine production in HBeAg-transgenic mice

In order to demonstrate the effect of EA on cytokine production in control, mice were primed by injection with HBeAg (2 μg) and boosted with 2 μg HBeAg after 2 weeks. Lymphocytes (1×10^6) were isolated and cultured in 96-well plates. After 0, 4 and 7 days, supernatants were collected and IL-4 and IFN-γ level was determined by ELISA. We have focused on IL-4 and INF-γ as the former is mainly produced by Th1 and the latter by Th2 cells, respectively. As shown in Fig. 6, EA stimulated IL-4 production in lymphocytes isolated from HBeAg-Tg mice at days 4 and 7, while IFN-γ production reached comparable level on day 7.

Groups of five control, HBeAg-Tg or EA fed HBeAg-Tg mice were immunized with HBeAg (2 μg) and boosted with 2 μg HBeAg, 2 weeks later. Ten days later, pooled spleen cells (1×10^6 cells per well) were collected and cultured with HBeAg (2 μg/ml) and cell supernatants were collected at day 4 and analyzed for IgM, IgA, IgG1, IgG2a, IgG2b and IgG3 anti-HBe antibodies by indirect solid-phase ELISA.

Specific cytolytic activity of splenocytes from HBeAg-immunized normal, HBeAg-Tg and EA fed HBeAg-Tg as effector cells and HBeAg-pulsed EL4 as target cells were determined. For the control, empty vector(pMT-SV40) transfected EL4 as targer cell was used. The data shown represent mean values plus

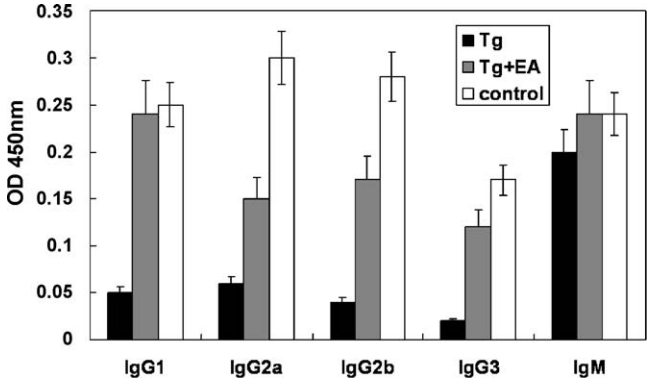

Fig. 4. In vitro anti-HBeAb production in C57ML/6 control, HBeAg-Tg and EA fed HBeAg-Tg mice.

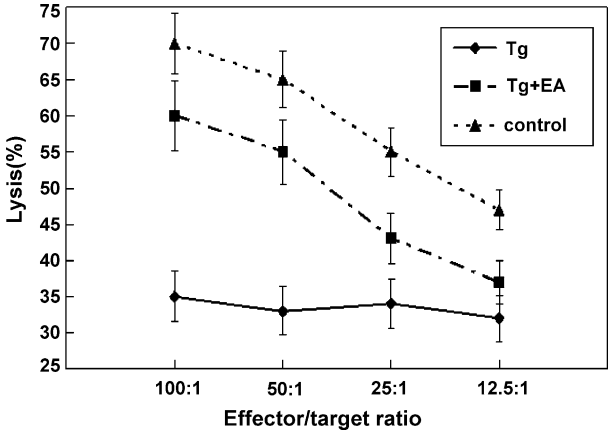

Fig. 5. EA effect on the CTL in HBeAg-Tg mice.

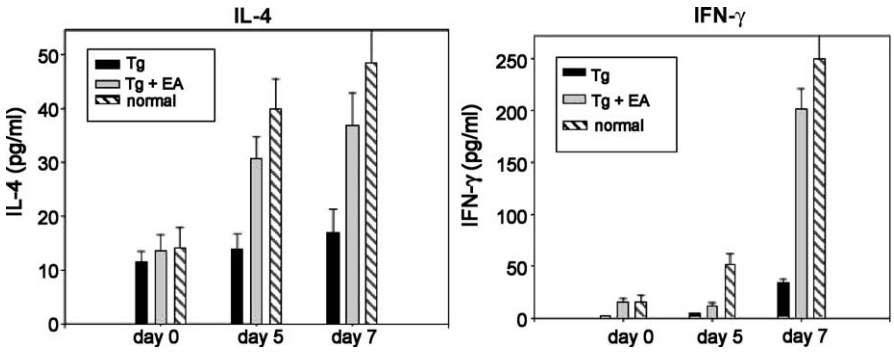

Fig. 6. EA-induced cytokine production in immune- tolerant HBeAg-Tg mice.

standard deviations of chrominium release assays performed with individual or pooled splenocytes.

Five mice of control, HBeAg-Tg and EA -fed HBeAg-Tg mice were primed with 2 μg of HBeAg and boosted 2 weeks later. Ten days later, the lymphocyte were harvested and 1×10^4 cells per well were cultured at 96-well plates. The presence of IL-4 and IFN-γ was determined at day 4 and day 7.

4. Discussions

A unique feature of the HBV is the production of a secreted, nonparticulate form of the nucleoprotein, designated hepatitis B precore Ag (HBeAg). The function of secretory HBeAg in the viral life cycle is unknown inasmuch as it is not required either for infection or for replication. Experimental results rather suggest that the HBeAg may modulate immune response during chronic infection in adults inaddition to the induction of neonatal tolerance. One way of examining the modulatory effect of secreted HBeAg on the immune system is to develop HBeAg-expressing Tg mice. We generated HBeAg-Tg mice to examine the effects of ellagic acid on the HBeAg specific immune tolerance response. Characterization of the HBeAg specific immune tolerance in C57ML/6 based HBeAg-Tg mice indicated that (i) T/B cells are made tolerant by HBeAg present in the serum (ii) HBeAg-Tg mice do not produce anti-HBe antibodies upon immunization (iii) the production of IgG subtype but not IgM, IgA subtypes of anti-HBeAb is attenuated in HBeAg-Tg mice. (iv) a decreased CTL response and cytokine (IL-2, IFN-γ) production were found in HBeAg-primed T-helper cells from HBeAg-Tg mice. The flavonoid EA, isolated from *Phyllanthus urinaria*, which was found to be a HBeAg secretion blocker (Shin et al., 2005), was tested for its inhibitory effect on the immune tolerance caused by HBeAg in HBeAg-Tg mice. Dietary supplements of EA (5 mg/kg body weight) to HBeAg-Tg mice resulted in decreased HBeAg levels and increased HBeAb production in sera, recovery of the T/B cell response, recovery of IgG-type antibody production, cytokine production and an increased CTL response. Collectively, EA ameliorated the immune tolerance caused by HBeAg in HBeAg-Tg mice. Since the induction of Th cell subsets can be examined by direct measurement of specific cytokine production and isotype analysis of antibodies (IgG and IgE production is regulated by IL-4 released from Th2 cells and IgG2a production is regulated by Th1-cell derived cytokine, IFN-γ, the release of the immune tolerance effects of HBeAg by ellagic acid on cytokine IFN-γ, production and antibody isotype production profiles were determined (Figs. 4 and 6). Since both the cytokine production and IgG subtype production of ellagic acid fed animal showed activation of Th1 and Th2 cells, the ellagic acid effect could occur on both subset of Th1 and Th2 cells or activation of Th_0 cell subsets. Other studies on HBeAg-Tg mice revealed that the level of Th cell tolerance was dependent on the MHC background and Th cell site recognized by the Tg murine strain (Milich et al., 1991). A some portion of Th cells of HBeAg-Tg mice in a H-2^b background (residues 129–140 specific) evaded tolerance induction and could be activated in vivo (Milich et al., 1991). Subsequent studies revealed that HBeAg-specific Th cells that evaded tolerance and mediate anti-HBe autoantibody production in HBeAg-Tg mice have been significantly 'altered' by the coexistence of circulating HBeAg. It was suggested that the self reactive Th cells that survived in HBeAg-Tg mice exhibited a unique fine specificity that can be distinguished from the HBeAg specific Th cell repertoire of non-Tg mice and comprised predominantly of Th2 like cells (Milich et al., 1995). The preferential survival of HBeAg-specific Th cells of the Th2-type in HBeAg-Tg mice is of particular interest because of the serologic evidence that an imbalance in HBe/HbcAg-specific Th1/Th2 cell function may contribute to the induction and/or maintenance of persistent HBV infection (Maruyama et al., 1993). Millich et al., also reported that circulating HBeAg has the potential to preferentially deplete inflammatory HBeAg and HBeAg-specific Th1 cells that are necessary for viral clearance, thereby promoting hepatitis B virus persistence (Millich et al., 1990). Further studies are needed to reveal the molecular mechanisms involved in relief of immune tolerance by EA administration in persistent HBV-infections. Effect of EA relief on (i) the specific MHC background of HBeAg-Tg mice (ii) the determination of Th1 and/or Th2 cells in evasion of immune tolerance (iii) specificity of HBeAg/self reactive Th cells in breaking immune tolerance.

Although EA exert various biological activities molecular mechanism response for the activities including anti-cancer properties (Li et al., 2005) remain largely unknown. But its potent scavenging action on both superoxide anion and hydroxyl anion might be involved in these processes. It has also been known that EA is well tolerated by both experimental animals and human (Bhargava et al., 1968; Lesca, 1983).

These observations, together with those of previous reports, indicate that immune tolerance caused by HBeAg with high chronicity rates (∼90%) observed in perinatally infected children born from their HBeAg-positive mothers, and many other cases (Holtby and Macarron, 2004), can be overcome by the use of EA isolated from medicinal herbs.

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