Dual immunomodulating effect of a traditional Japanese medicine, *Hachimi-jio-gan* (Ba-Wei-Di-Huang-Wan), on Th1/Th2 balance in both the adjuvant-induced Th1- and Th2-predominant immune responses

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Abstract

Complete Freund's adjuvant (CFA) induced a Th1-predominant immune response, accompanied by a high level of antigen (Ag)-specific IgG2a and a low level of Ag-specific IgE. Administration of *Hachimi-jio-gan* (HMG : Ba-Wei-Di-Huang-Wan), a traditional Japanese herbal medicine (*Kampo*) originating in China, for 1 week before and 2 weeks after immunization, elicited the suppression of Ag-specific IgG2a and the enhancement of Ag-specific IgE in the mice immunized with CFA. Ag-specific IFN-γ production was suppressed by HMG, whereas Ag-specific IL-4 production showed a tendency for augmentation by HMG. Additionally, anti-CD3-stimulated IFN-γ and IL-4 production during the primary antigenic stimulation of naive T cells, was NK1.1 cell-dependently inhibited and augmented by HMG, respectively. On the other hand, aluminum hydroxide (alum) induced a Th2-predominant immune response, accompanied by a low level of Ag-specific IgG2a and a high level of Ag-specific IgE. Treatment with HMG for 2 weeks after immunization, elicited the suppression of Ag-specific IgE in mice immunized with alum. Ag-specific IFN-γ production was clearly up-regulated by HMG.

These results suggest that HMG has dual regulatory effects on Th1/Th2 balance in both the adjuvant-induced Th1- and Th2-predominant immune responses through IFN-γ and IL-4 productions from NK1.1 cells during the primary antigenic stimulation of naive T cells.

Key words Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan, 八味地黄丸), CFA, alum, NK1.1.

Abbreviations Ag, antigen; alum, aluminum hydroxide; CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; HMG, Hachimi-jio-gan; LIV, liver; MNC, mononuclear cell; MoAb, monoclonal antibody; NKT, natural killer T cell; OVA, ovalbumin; SPL, spleen; Th, T helper.

Introduction

MRL/MpJ-*lpr*/*lpr* (MRL/*lpr*) mice have been used as a model of systemic autoimmune disease characterized by auto-antibody, immune-complex formation, and massive lymphadenopathy or splenomegaly.[1)] In the mice, the following immunological abnormalities are observed ; a defect in the activation-induced cell death of peripheral T cells,[2,3)] a skewing of the immune response towards T helper (Th) 1 cells,[4)] a hyper-responsiveness of cells to IL-18,[5)] a reduction of Vα14 NKT cells,[6)] and the like. Our previous studies have shown that *Hachimi-jio-gan* (HMG : Ba-Wei-Di-Huang-Wan), a traditional Japanese herbal medicine (*Kampo*) originating in China, ameliorates autoimmune diseases in MRL/*lpr* mice through the reduction of Th1 predominance,[7,8)] suppression of the IL-18 hyper-responsiveness and an increase in Vα14 natural killer T (NKT) cells.[9)] Since Vα14 NKT cells can secrete large amounts of IFN-γ and IL-4,[10,11)] a regulatory role for these cells in Th1/Th2 differentiation has been suggested. Therefore, it has been speculated that the suppressive effect of HMG on the development of MRL/*lpr* autoimmune diseases is mediated by Vα14

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NKT cells. In the present study, normal mice were injected intraperitoneally (i.p.) with Complete Freund's adjuvant (CFA) to induce an imbalance toward Th1 predominance and with aluminum hydroxide (alum) to induce an imbalance toward Th2 predominance. CFA promotes the synthesis of antigen (Ag)-specific IgG1 and IgG2a in response to T-dependent antigens,[12,13] but IgE was detected at low levels in the serum after CFA treatment. On the other hand, alum promotes the production of Ag-specific IgG1 and IgE.[14] In these models, we investigated the immunomodulating effect of HMG on the Th1/Th2 balance.

As a result, we demonstrated that HMG had a dual regulatory effect on Th1/Th2 balance in both the Th1-predominant immune response to CFA and Th2-predominant immune response to alum.

Materials and Methods

Experimental animals: Female C57BL/6 mice (7 weeks old) were purchased from Charles River Japan Inc.(Yokohama, Japan), and maintained in specific pathogen-free facilities.

Immunization of mice: Ovalbumin antigen (OVA) (gradeV SIGMA, St. Louis, MO) was emulsified in Complete Freund's adjuvant (CFA)(Difco Laboratories, Detroit, MI). Groups of 4 ~ 6 mice at 9 weeks of age were immunized i.p. with 100μg of OVA in CFA (CFA-OVA) at a volume of 0.2ml. In another experiment, groups of 5 mice at 9 weeks of age were immunized i.p. with 4μg of OVA absorbed on 4mg of alum (Wako, Osaka) (alum-OVA), suspended in 0.2ml of phosphate-buffered saline (PBS).

Treatment with HMG (Hachimi-jio-gan): A spray-dried HMG extract manufactured at Kanebo Ltd. (Tokyo, Japan) was used as the test drug (human's usual dose ; 5.2g/day). HMG was prepared from a mixture of 8 medical herbs in the following proportions: *Rehmanniae radix* (5.0 g), *Corni fructus* (3.0 g), *Dioscoreae rhizoma* (3.0 g), *Alismatis rhizoma* (3.0 g), *Poria* (3.0 g), *Moutan cortex* (3.0 g), *Cinnamomi cortex* (1.0 g), and processed *Aconitum tuber* powder (1.0 g). Extraction was carried out by refluxing one part herb mixture with ten parts water at 95 ~ 100 ℃ for 1 hr. The extract was then spray-dried in a hot air stream (Recovery : about 22%).

C57BL/6 mice were orally administered 1000 mg/kg of HMG suspended in distilled water for 1 week before CFA-OVA immunization (termed before-HMG ; -day 7 ~ -day 1), 2 weeks after the immunization (termed after-HMG ; day 0 ~ day 13) or 3 weeks throughout (termed all-HMG ; -day 7 ~ day 13). In the alum-OVA experiment, C57BL/6 mice were orally administered 1000mg/kg of HMG after alum-OVA immunization (day 0 ~ day 13).

Measurement of OVA-specific IgE and IgG subclasses in serum: Detection of OVA-specific IgG1, IgG2a, and IgE in the serum 14 days after immunization was carried out using an enzyme-linked immunosorbent assay (ELISA). For the measurement of OVA-specific IgG1 and IgG2a, a plate was coated with 10 and 100 μg/ml of OVA, respectively. Then, OVA-specific IgG1 and IgG2a bound to the plate were detected by peroxidase(POD)-conjugated anti-mouse IgG1 and IgG2a MoAbs (LO-MG1-2, LO-MG2a-3, ZYMED Inc. San Francisco, CA), respectively. The values were measured at 492 nm with a microplate reader (Em ; 630nm). For the measurement of OVA-specific IgE, a plate was coated with purified anti-mouse IgE (LO-ME-2, Serotec, CA). Then, OVA-specific IgE bound to the plate was detected by biotin-OVA, followed by streptavidin conjugated β-galactosidase (CALTAG, CA). After the substrate reaction using 4-methylumbelliferyl β-D-galactopyranoside (Wako), a stop solution was added, and optical fluorescence intensities were measured with a microplate reader at 360nm (Em ; 450nm).

Cell preparation and separation: Spleen (SPL) cells obtained from each group were mixed and suspended in RPMI 1640 medium (Invitrogen, Groningen, Netherlands) supplemented with 10% fetal bovine serum (Invitrogen), 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (Dojindo, Kumamoto, Japan), 2×10^{-5} M 2-mercaptoethanol and 10 mg/l of gentamicin sulfate. Liver (LIV) mononuclear cells (MNCs) were isolated by Percoll (SIGMA) density gradient centrifugation as previously described[14] and suspended in the RPMI 1640 medium. $NK1.1^+$ cell-depleted SPL cells were obtained using the magnetic beads conjugated with anti NK1.1 MoAb (Miltenyi Biotec, Germany).

Proliferative activity: SPL cells (2.5×10^5 /well) obtained 14 days after CFA or alum immunization were seeded into each well of 96-well plates, and incubated for 72 hr with 5 and 50 μg/ml (CFA), or 50 and

500 μg/ml (alum) of soluble OVA. Cell proliferation was detected by Cell Proliferation ELISA BrdU (Roche, Mannheim) at 450nm (Em ; 630nm).

Cytokine production: SPL cells (2.5×10^5 /well) obtained 14 days after CFA or alum immunization were incubated for 72 or 96 hrs in non-coated 96-well plates with 500 μg/ml of soluble OVA, respectively. $NK1.1^+$-depleted SPL cells ($NK1.1^-$ cells) and whole SPL cells (Whole cells) obtained day 1 after CFA immunization were cultured in 96-well plates pre-coated with 10 μg/ml αCD3 MoAb for 48 hrs. Detection of IFN-γ and IL-4 in the culture supernatants was carried out by ELISA. Recombinants of IFN-γ (Pharmingen) and IL-4 (Pharmingen) were used as standards. Each cytokine was captured by pre-coated anti-Mouse IFN-γ (R4-6A2, Pharmingen) and anti-Mouse IL-4 (BVD4-1D11, Pharmingen) MoAbs, respectively. Then, each captured cytokine was detected by biotin-conjugated anti-Mouse IFN-γ MoAb (XMG1.2, Pharmingen) or biotin-conjugated anti-Mouse IL-4 MoAb (BVD6-24G2, Pharmingen), followed by streptavidin-conjugated β-galactosidase (CALTAG) and 4-methylumbelliferyl β-D-galactopyranoside (Wako). After the substrate reaction, a stop solution was added and optical fluorescence intensities were measured with a microplate reader at 360 nm (Em ; 450nm).

Statistical analysis: Data was expressed as the mean ± S.D. The statistical significance of difference was analyzed with Student's *t*-test or Mann-Whitney's *U*-test.

Results

Effect of HMG on OVA-specific IgE, IgG1 and IgG2a levels in serum of CFA-OVA-immunized mice

Immunization with CFA-OVA strongly induced the production of OVA-specific IgG1 and IgG2a, whereas very little OVA-specific IgE was detected in the serum of C57BL/6 mice (Fig.1, control). The mice were orally treated with 1000 mg/kg of HMG for 1 week before CFA-OVA immunization (before), 2 weeks after the immunization (after) or 3 weeks throughout (all). The treatment with HMG did not affect OVA-specific IgG1 levels, regardless of the difference in schedule (Fig.1-A). As shown in Figure 1-B, all-HMG significantly reduced OVA-specific IgG2a levels, though the partial treatment (before, after) did not rise to the significant suppression of the level. Moreover, the all-HMG treatment in CFA-OVA mice increased OVA-specific IgE levels, and differences in the effect among schedules were similar to those in the case of OVA-specific IgG2a (Fig.1-C). Judging from these results, we selected "all" as a treatment schedule in the next experiment.

Suppressive effect of HMG on Th1 immune response

To examine the effects of HMG on Th1 and Th2 cell development, SPL cells were obtained from the mice 14 days after immunization. The production of IFN-γ in the culture supernatants of OVA-stimulated SPL cells

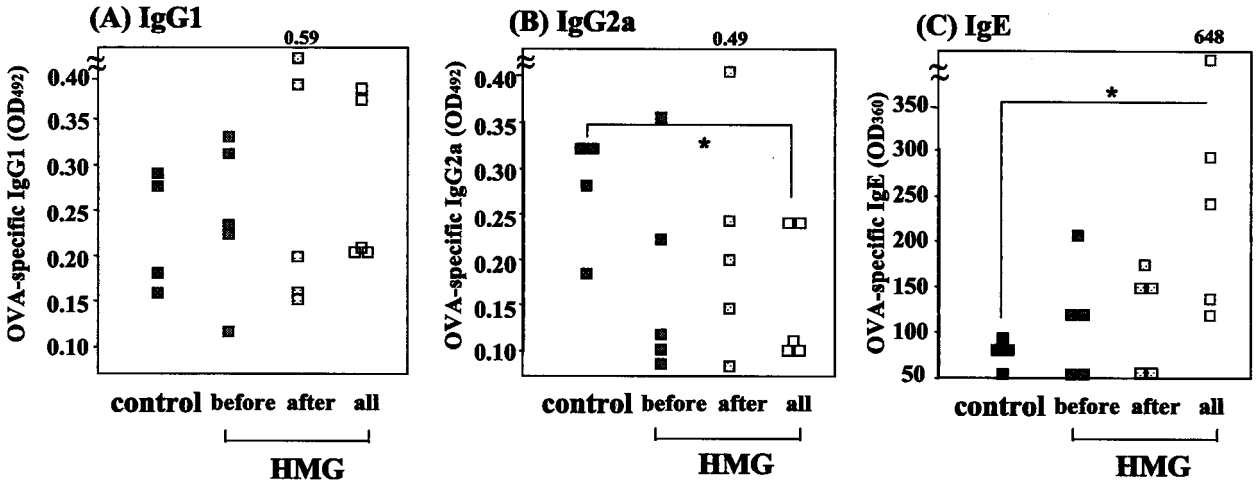

Fig. 1 Effect of HMG on OVA-specific IgE, IgG1 and IgG2a levels in serum of CFA-OVA immunized mice.
C57BL/6 mice were i.p. immunized with 100μg of OVA in CFA on day 0. The mice were orally administered 1000mg/kg of HMG on day-7~1 (before), day 0~13 (after) or day-7~13 (all). OVA-specific IgG1 (A), IgG2a (B) and IgE (C) levels in serum on day 14 were determined by ELISA (see Materials & Methods). The statistical significance of differences was analyzed with Mann-Whitney's *U*-test. *, $p<0.05$ compared with control

was significantly suppressed by "all" HMG treatment (Fig.2). The production of IL-4 showed a tendency for augmentation by HMG treatment. On the other hand, the proliferation of SPL cells in response to OVA was significantly suppressed by HMG (data not shown).

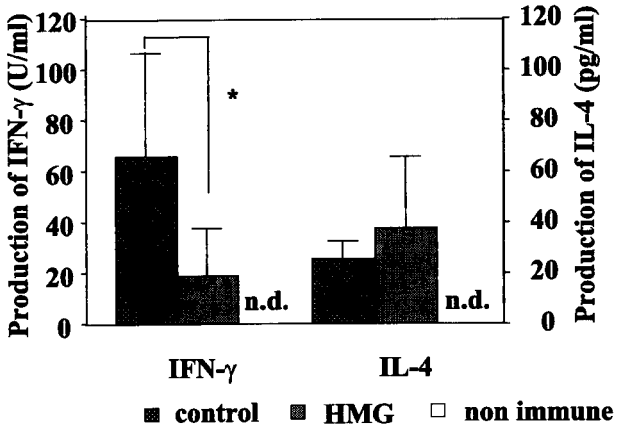

Fig. 2 Suppressive effect of HMG on Th1 immune response.
C57BL/6 mice were i.p. immunized with 100μg of OVA in CFA on day 0. The mice were orally administered 1000mg/kg of HMG on day-7 ~13. On day 14, SPL cells (2.5 x 10^5/well) were pooled from 4 ~6 mice in each group and cultured with 500 μg/ml of OVA for 72 hr. The production of IFN-γ (U/ml) and IL-4 (pg/ml) in the supernatants was measured by ELISA (see Materials & Methods). Data represent the mean ± S.D. at OD_{360}. The statistical significance of differences was analyzed with Student' *t*-test. *, $p<0.05$ compared with control. n.d.= not detected

Effect of HMG on NK1.1-positive cell produced cytokines regulating subsequent Th1/Th2 differentiation in CFA-OVA immunized mice

In the early period of immunization, cytokines produced by NK1.1-positive cells have an important role in subsequent Th1/Th2 differentiation. Especially, NKT cells can secrete large amounts of IFN-γ and IL-4 in response to stimulation.[10,11] To establish the effect of HMG on cytokine produced in early period of CFA-OVA immunization, the production of IFN-γ and IL-4 in culture supernatants of αCD3-stimulated SPL cells obtained the day after immunization and the last HMG treatment, were examined (Fig.3-A,B). Additionally, the production of IFN-γ and IL-4 was examined using NK1.1-depleted SPL cells ($NK1.1^-$ cells) in place of whole SPL cells (Whole cells). Whole cells in immunized control mice produced fairly large amounts of IFN-γ and IL-4 (Fig.3-A,B). The production depended on NK1.1 cells, because $NK1.1^-$ cells produced only a small amount of IFN-γ or IL-4. However, non-immunized SPL cells (non immune) produced considerable amounts of IFN-γ, but most of such IFN-γ production was NK1.1 cells-independent. There was NK1.1 cell-independent IL-4 production. The production of IFN-γ in Whole cells was significantly inhibited by HMG, whereas IL-4 pro-

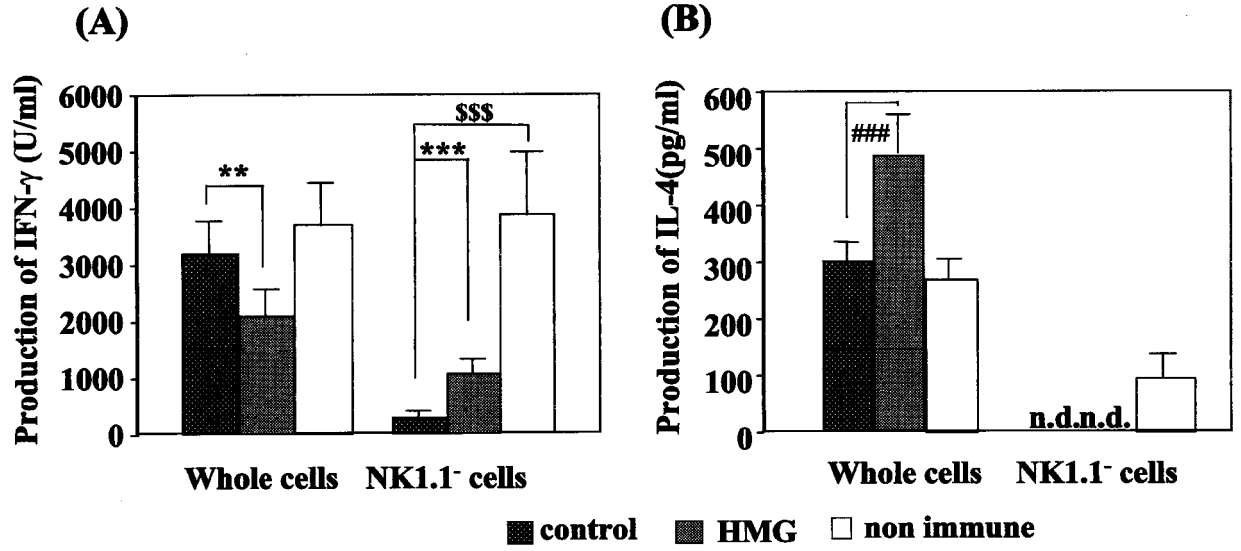

Fig. 3 Effect of HMG on NK1.1-positive cell produced cytokines regulating subsequent Th1/Th2 differentiation in CFA-OVA immunized mice.
C57BL/6 mice were i.p. immunized with 100μg of OVA in CFA on day 0. The mice were orally administered 1000mg/kg of HMG on day-7 ~0. On day 1, SPL cells (Whole cells) were pooled from 4~6 mice in each group and $NK1.1^+$ cell-depleted SPL cells ($NK1.1^-$ cells) were obtained from Whole cells (see Materials & Methods). These cells (2.5 x 10^5/well) were cultured with pre-coated αCD3 MoAb for 48 hrs. The production of IFN-γ (A) and IL-4 (B) in the supernatants was measured by ELISA (see Materials & Methods). Data represent the mean ± S.D. of concentration (U/ml, pg/ml). The statistical significance of differences was analyzed with Student' *t*-test.***, $$$, ###, $p<0.001$; **, $p<0.01$ compared with control. n.d.= not detected

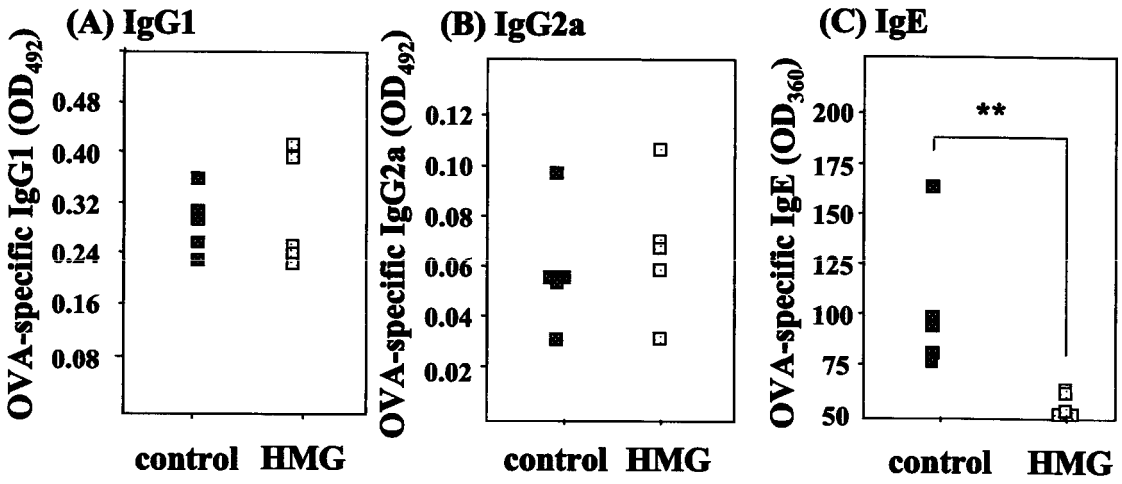

Fig. 4 Effect of HMG on OVA-specific IgE, IgG1, and IgG2a levels in serum of alum-OVA immunized mice.
C57BL/6 mice were i.p. immunized with 4 μg of OVA in 4 mg of alum on day 0. The mice were orally administered 1000mg/kg of HMG on day 0~13. OVA-specific IgG1 (A), IgG2a (B) and IgE (C) levels in serum on day 14 were measured by ELISA (see Materials & Methods). The statistical significance of differences was analyzed with Mann-Whitney's *U*-test. **, $p<0.01$ compared with control.

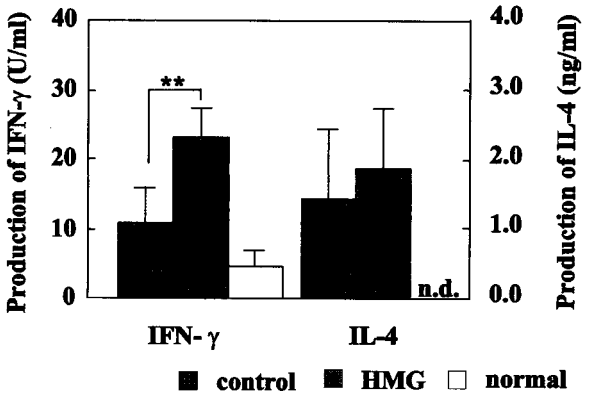

Fig. 5 Suppressive effect of HMG on Th2 immune response.
C57BL/6 mice were i.p. immunized with 4 μg of OVA in 4mg of alum on day 0. The mice were orally administered 1000mg/kg of HMG on day 0~13. On day 14, SPL cells (2.5 x 10^5/well) were pooled from 4~6 mice in each group and cultured with 500 μg/ml of OVA for 96 hr. The production of IFN-γ (U/ml) and IL-4 (ng/ml) in the supernatants was measured by ELISA (see Materials & Methods). Data represent the mean ± S.D. at OD_{360}. The statistical significance of differences was analyzed with Student' *t*-test. **, $p<0.01$ compared with control. n.d.= not detected

duction was significantly promoted. The production of IL-4 depended on NK1.1 cells, though in the case of IFN-γ, NK1.1 cell-independent production remained. These results indicated that HMG regulated Th1/Th2 differentiation, mediated by NK1.1 cell-dependent cytokine production.

Effect of HMG on OVA-specific IgE, IgG1 and IgG2a levels in serum of alum-OVA-immunized mice

On the other hand, the effect of HMG on the Th2 immune response was also examined. Immunization with alum-OVA in place of CFA-OVA strongly induced production of OVA-specific IgE in C57BL/6 mice. As shown in Figure.4, the treatment with HMG significantly reduced OVA-specific IgE levels, though HMG had almost no effect on OVA-specific IgG1 and IgG2a levels.

Suppressive effect of HMG on Th2 immune response

Likewise, we examined the effects of HMG on Th1 and Th2 cytokine productions from SPL cells of mice on day 14 after alum immunization. The production of IFN-γ in the culture supernatants of OVA-stimulated SPL cells was significantly increased by HMG treatment (Fig.5). The production of IL-4 showed no change by HMG treatment. On the other hand, there was no difference in the proliferative response of SPL cells to OVA between control and HMG group (data not shown).

Discussion

In the present study, we established that the dual regulatory effect of HMG achieved Th1/Th2 balance in both the Th1-predominant immune response to CFA-OVA, and Th2-predominant immune response to alum-OVA. HMG causes a swing back to Th2 from the Th1-predominance not only in autoimmune MRL/*lpr* mice as previously reported,[7,8] but also in normal strain mice. HMG has a regulatory effect on Th1/Th2 differentiation is confirmed by HMG-induced changes in opposite

directions of OVA-specific IgG2a and IgE levels (Fig.1-B, C), or IFN-γ and IL-4 production (Fig.2). However, HMG regulates Th1/Th2 differentiation in opposite directions depending on the Th1/Th2 balance. Th1 and Th2 cells are thought to be differentiated from a common precursor and the direction of Th cell differentiation into Th1 and Th2 cells is dependent on exogenous cytokines present during the primary antigenic stimulation of naive T cells. It is now well proved that the potent inducer of Th1 is IL-12.[17] IFN-γ is reported to induce IL-12 production from various antigen presenting cells and develop Th1 cells.[18,19] Additionally IFN-γ inhibits development of Th2 cells.[17,20] On the other hand, IL-4 is required for the differentiation of naive T cells into Th2 effector cells.[21] The production of IFN-γ in αCD3-stimulated SPL cells obtained the day after immunization was significantly inhibited by HMG, whereas IL-4 production was significantly promoted (Fig.3). In immunized mice treated with or without HMG, their IFN-γ and IL-4 productions were depended on NK1.1-positive cells. To explain the mechanism of action of HMG, the participation of NK1.1-positive cells is suggested. Especially, NKT cells represent a unique lymphoid lineage and consist of a single invariant Vα14-Jα281 chain and Vβ8.2, Vβ7, or Vβ2[22,23] can secrete large amounts of IFN-γ and IL-4 by αCD3 or α-galactosylceramide (α-GalCer).[10,11] Therefore, they can regulate Th1/Th2 balance in opposite directions. NKT cells are not essential for the induction of IgE responses but rather induce suppression of specific IgE production upon activation.[24,25] Coadministration of α-GalCer and protein Ag in CFA promotes the differentiation of Ag-specific CD4 T cells into Th2 cells, which results in a profound increase in Ag-specific IgE Ab.[26] The regulatory effect of HMG on cytokine productions from NK1.1 cells, presumably Vα14NKT cells in early period of immunization is suggested to lead to the change of OVA-specific antibody in Th1-, or Th2-predominance, though the evidence for this is incomplete. Since HMG prevents the loss of Vα14NKT cells which play a crucial role in controlling the development of autoimmune diseases in MRL/*lpr* mice, HMG may activate Vα14NKT cells.

In conclusion, HMG has dual regulatory effect on Th1 /Th2 balance in both the Th1-predominant immune response to CFA-OVA and Th2-predominannt immune response to alum-OVA. In the effect of HMG, participation of cytokine productions from NK1.1 cells, presumably Vα14NKT cells, in early period of immunization is suggested though further study is needed to obtain clear-cut evidence.

和文抄録

フロインドの完全アジュバント（CFA）は，抗原特異的な高 IgG2a 産生および低 IgE の産生を伴い，Th1 優位な免疫反応を誘導する。漢方方剤である八味地黄丸（HMG）を，CFA 免疫前 1 週間および免疫後 2 週間経口投与すると，CFA 免疫マウスの抗原特異的な IgG2a 産生が低下し，IgE 産生が亢進した。抗原特異的な IFN-γ 産生も HMG 投与により有意に抑制されたが，IL-4 については増加傾向にとどまった。さらに，CD3 抗体を用いて，ナイーブ T 細胞への一次抗原刺激を行った場合の IFN-γ および IL-4 産生は，NK1.1 細胞依存的にそれぞれ減少（IFN-γ）と増加（IL-4）を示した。一方，水酸化アルミニウム（alum）は，抗原特異的な高 IgE 産生と低 IgG2a 産生を伴い，Th2 優位な免疫反応を誘導する。alum 免疫後に 2 週間 HMG を投与すると，抗原特異的な IgE の産生抑制が誘導された。抗原特異的な IFN-γ 産生は HMG 投与により著しく増加した。これらの結果から，HMG は，ナイーブ T 細胞への一次抗原刺激時に，NK1.1 細胞からの IFN-γ および IL-4 産生を介して，アジュバントによって誘導された Th1 および Th2 優位な免疫反応の両方において二面的な調節作用を有することが示唆された。

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