Administration of Ag85B showed therapeutic effects to Th2-type cytokine-mediated acute phase atopic dermatitis by inducing regulatory T cells

Running title: Ag85B ameliorates Th2 type -mediated dermatitis.

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Conflicts of interest: none
Word count: 3789
Character count: 20911
Key words: atopic dermatitis, antigen 85B, regulatory T cell
Abbreviations: AD, atopic dermatitis; Ag85B, antigen 85B; OX, oxazolone; Treg, regulatory T cell

Abstract Increase in the number of patients with atopic dermatitis (AD) has been recently reported. T helper (Th) cells that infiltrate AD skin lesions are Th2-type dominant; reduced exposure to environmental Th1-cytokine-inducing microbes is believed to contribute to the increased number of AD patients. Regulatory type immune responses have been also associated with the occurrence of AD. It has been reported that Ag85B purified from *Mycobacteria* is a potent inducer of Th1-type immune response in mice as well as in humans. In this study, we have examined the effect of plasmid DNA encoding Ag85B derived from *Mycobacterium kansasii* on AD skin lesions induced by Oxazolone(?) application. Th2-cytokine mediated mouse AD model with immediate type response followed by a late phase reaction was developed by repeated applications of low-dose oxazolone to sensitized mice. Mice were immunized with plasmid DNA enocoding cDNA of Ag85B before OX sensitization or during repeated elicitation phase. Both therapies were associated with significant suppression of immediate type response, clinical appearance, dermal cell infiltration, reduced IL-4 production and augmented IFN-γ mRNA expression compared to placebo-treated mice. Additionally, increased number of $Foxp3^{+}$ regulatory T cells were observed in the cervical lymph nodes in Ag85B treated mice. The results of this study suggest that Ag85B DNA vaccine is a potential therapy for Th2 type dermatitis.

Introduction

It is known that acute phase skin lesion in atopic dermatitis (AD) is associated with enhanced secretion of T helper (Th) 2-type cytokines [8]. Increased incidence of atopic disorders has been reported in industrialized countries; according to the hygiene hypothesis, the increase in the incidence of patients may be explained by a better lifestyle and less exposure to environmental microbes [5, 7, 28]. Environmental microbes such as *Mycobacteria* or certain virus may promote Th1-type immune response and thus reducing atopy-associated Th2-type reaction. For instance, the study carried out in Japanese *Bacillus Calmette-Güerin* (BCG)-vaccinated school children showed that responders to tuberculin had a lower prevalence of atopic disease compared totuberculin non-responders [28]. BCG-treated mice showed suppression of experimental allergic responses [12]. More recently, it has been shown that microbial stimulation can induce regulatory T (Treg) cells with the ability to suppress both Th1-type and Th2-type inflammation [35]. In the experimental model of pulmonary inflammation, *Mycobacterium vaccae* reduces allergic pulmonary inflammation significantly by increasing the number of Treg cells that secretes IL-10 and TGF- β [37]. These observations indicate that shift from Th2 to Th1 type immune response by *Mycobacteria* may be used for the prevention and treatment of atopic disorders.

The specific antigens eliciting Th1-type immune responses in *Mycobacteria* have not been elucidated so far; a recent study suggested that one of the specific proteins for Th1 development is antigen 85B (Ag85B) [31]. Ag85B is a 30k-Da major protein secreted from all mycobacterium species and that belongs to the Ag85 family[4]. The Ag85B can induce a strong Th1-type immune response in mice as well as in humans [31], and DNA vaccines encoding Ag85B have been reported to protect animals from tuberculosis infection by inducing Th1 response [34, 36]. We have previously reported enhancement of anti-tumor specific CTL response using Ag85B-transfected tumor cells, and by inducing Th1-type immune responses as a vaccine adjuvant [22, 30].

The purpose of the present study was to evaluate the therapeutic efficacy of Ag85B derived from *Mycobacterium kansasii* in acute phase dermatitis. Repeated applications of hapten such as oxazolone (OX) on BALB/c mice causes delayed type hypersensitivity in the beginning that changes to an immediate-type response in the late phases with elevated IgE production, and deviation of Thcell responses. The skin

lesions that appear in late phases are compatible with the clinical findings as well as cytokine profile observed in AD [19, 20]. In all Ag85B-treated AD mice, the immediate type reaction is effectively suppressed and IL-4 is significantly reduced. The results of this study provide evidence for the potential usefulness of Ag85B as a novel approach for the treatment of Th2 type-mediated dermatitis such as AD.

Materials and methods

Animals

6-week-old BALB/c male mice were purchased from Japan SLC Co. (Shizuoka, Japan) and used at the age of 7 weeks. Animal care was done according to ethical guidelines, and approved by the Institutional Board Committee for Animal Care and Use of Mie University.

Sensitization and challenge of animals

OX was purchased from Sigma (St. Louis, MO), and dissolved in acetone/olive oil (4:1). As shown in Fig.1, mice were initially sensitized by pasting 20 μ l of 0.5% OX solution to their left ear 7 days prior to the first challenge (day -7) and then 20 μ l of 0.5% OX solution was repeatedly applied on the left ear 3 times per week from day 0. The ear swelling response was expressed as the difference between before and 30min after application. The Ag85B expression vector pcDNA-Ag85B of *Mycobacterium kansasii* open reading frame lacking a signal sequence has been constructed into Kpnl-Apa I sites of pcDNA3.1 as described previously [22]. Plasmid DNAs were purified using the Plasmid Mega Kit (Qiagen, Chatsworth, CA). The empty plasmid pcDNA3.1 was used as a control. Plasmid DNAs were diluted with sterilized physiological saline. 100 μ g/ mouse of plasmid DNA was injected intraperitoneally on day-14, -7 to evaluate prophylactic effects, or on day 14 and 21 for the assessment of therapeutic effects.

Histological analysis

Skin specimens obtained 30 minutes after the final challenge were fixed in 10 % buffered neutral formaldehyde and embedded in paraffin. Sections prepared of 7μm thickness were stained with hematoxylin and eosin (H&E), or truidine blue.

Immunohistochemistry

The left ear was sacrificed on day 35, and was embedded in Tissue-Tek OCT compound (Miles, Elkhart, USA), frozen in liquid nitrogen, and cut with a cryostat into 7 μm-thick sections. The tissue preparations were then incubated with primary antibodies specific for Foxp3 (eBioscience, San Diego) overnight, followed by the additional incubation with Alexa Fluor 633 conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 30 minutes at room temperature. Sections were examined under Fluoview FV1000 laser scanning confocal microscopy (Olympus, Tokyo, Japan). The numbers of $Foxp3^+$ cells were counted in high power fields; five randomly chosen fields were evaluated.

Analysis of cytokine mRNA expression in mouse ears

At 6 hours after the final challenge, the left ear skin was sampled. The specimen was homogenized and mRNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacture's instruction; one milliliter of homogenate was vigorously mixed with 200 μl of chloroform, and then centrifuged at 15000 r.p.m. for 15 min at 4℃. Aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifugation, the precipitate was washed with 1 ml of 75% ethanol (Nacalai Tesque) and dried up. RNA was suspended in 50 μL of RNase-free water, the concentration was calculated based on the absorbance at 260 nm, and the quality was confirmed by electrophoresis. cDNA was synthesized from 10 μg of mRNA using archive kit (ABI, Foster City, CA) according to the manufacturer's protocol.

Cytokine mRNA expression in skin

Real time quantitative RT-PCR was performed to measure transcriptional activity in the skin lesions. A 25 μl reaction mixture containing 1 μg total of cDNA, 900 nmol of each primer, and 250 nmol of TaqMan probe were mixed with 12.5 μl of TaqMan Master Mix (ABI, Foster City, CA). The following primers and probes were used for the PCR reactions: mouse IL-4; forward: 5'-ACAGGAGAAGGGACGCCAT-3', reverse: 5'-GAAGCCCTACAGACGAGCTCA-3', probe: 5'-TCCTCACAGCAACGAAGAACACCACA-3'-TAMRA, IFN-γ; forward: 5'-TCAAGTGGCATAGATGTGGAAGAA-3', reverse:

5'-TGGCTCTGCAGGATTTTCATG-3', probe: 5'-TCACCATCCTTTTGCCAGTTCCTCCAG-3'-TAMRA, IL-10; forward: 5'-GGTTGCCAAGCCTTATCGGA, reverse: 5'-ACCTGCTCCACTGCCTTGCT, probe: 5'-TGAGGCGCTGTCGTCATCGATTTCTCCC-TAMRA, TGF-β; forward: 5'-TGACGTCACTGGAGTTGTACGG, reverse: 5'-GGTTCATGTCATGGATGGTGC, probe: 5'-TTCAGCGCTCACTGCTCTTGTGACAG-TAMRA, β-actin; forward: 5'-AGAGGGAAATCGTGCGTGAC-3', reverse: 5'-CAATAGTGATGACCTGGCCGT-3', probe: 5'-CACTGCCGCATCCTCTTCCTCCC-3'-TAMRA [25]. PCR was performed under the following conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60℃ for 1 min were carried out. Fluorescence data were collected during each annealing-extension step and analyzed by using ABI Prism SDS software version 1.9.1. All samples were normalized for to the β-actin mRNA content.

Measurement of serum IgE

Blood was collected under anesthesia 6 hours after the last challenge. Serum IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (BD PharMingen, California, USA) according to the manufacturer's instructions. Optical density of each well was determined by using a microplate reader (Multiscan JX) (Thermo Electron, Yokohama, Japan). Standard curve was prepared using mouse anti-TNP IgE standard (BD PharMingen, California, USA) diluted with PBS containing 10% FCS.

Statistical analysis

Differences in ear swelling and serum IgE levels were analyzed by the Kruskal-Wallis test. $P < 0.05$ was taken as significant.

Results

Effect of Ag85B on skin inflammation

We first examined whether Ag85B could modulate ear-swelling reaction in a mouse model of OX-induced atopic dermatitis. Repeated applications of OX cause Th2-mediated immediate type response. Ear swelling was measured with thickness gauge calipers before and 30 minutes after OX challenge on the pinna of the ear on day 32. In both prophylactic and therapeutic models, the administration of Ag85B

significantly suppressed swelling compared to placebo-treated controls (Fig. 2a). The OX-challenged placebo-treated mice showed severe skin inflammation, however administration of Ag85B DNA reduced atopic inflammatory reactions (Fig. 2b).

Histological analysis

Histological examination in OX-challenged mice showed epidermal hyperplasia and strong intra-epidermal and intra-dermal inflammatory cell infiltration including mononuclear cells, neutrophils, and granular cells (Fig 3a). Both prophylactic and therapeutic administration of Ag85B DNA clearly reduced inflammatory cell infiltration and epidermal thickness.. Skin sections stained with truidin blue showed decreased mast cell infiltration in Ag85B-treated mice (Fig.3b).

Ag85B treatment shifted the Th1/Th2 balance towards Th1

IFN-γ and IL-12 shift the Th1/Th2 balance toward Th1 condition; while IL-4 and IL-5 are key cytokines in Th2 response [24, 29]. To clarify the type of immune response in skin lesions after treatment with Ag85B, we analyzed the mRNA expression levels of IL-4 and IFN-by real time quantitative reverse transcription-polymerase chain reaction (RT-PCR). The results were normalized to the β-actin mRNA content. As shown in Fig 4, the expression of IL-4 mRNA was reduced in Ag85B-treated mice in both prophylactic and therapeutic models. On the contrary, the expression of IFN-γ was enhanced in Ag85B-treated mice. These results suggest that the application of Ag85B shifts the immune response towards Th1-predominace.

Total serum IgE levels

AD is characterized by elevated IgE levels. Repeated applications of OX cause a gradual elevation of antigen-specific IgE level. We analyzed the degrees of IgE levels in sera collected from experimental mice. Administration of Ag85B significantly reduced the serum levels of IgE (Fig 5).

Ag85B treatment induces regulatory T cells

TGF-β and IL-10 are important regulatory cytokines produced by Treg [11]. To investigate the mechanisms of the therapeutic effectiveness of Ag85B, we examined the

mRNA levels of TGF-β and IL-10. As shown in Fig. 6a, TGF-β and IL-10 were significantly increased in Ag85B-treated mice in both prophylactic and therapeutic models. And then, we next looked at the induction of Treg in the inflamed skin. Naturally occurring $CD4^+CD25^+$ Treg are characterized by the expression of Foxp3 [10, 27]. Skin sections were stained with anti-Foxp3 mAb, and examined with a fluorescent microscope. As shown in Fig. 6b, $Foxp3^+$ cells were increased in the Ag85B-treated mice.

Discussion

Human immune system responds to exogenous microorganisms for self-protection. These responses lead to Th1 and /or Th2 type cytokine secretion depending on the nature of stimuli. Atopic dermatitis is a chronic dermatitis characterized by a Th2-type immune responses that causes elevation of IgE. On the other hand, some bacterial infections including mycobacterium species elicits strong Th1-type responses. Inducers of Th1 type immune response may be used as immuno-modulator having therapeutic effects against allergic disease elicited by Th2-type immune responses. Mycobacteria may affect atopic disorders by correction of the immune response from Th2 to Th1. Erb KJ *et al* reported that *mycobacterium bovis* (BCG) suppresses airway eosinophilia and associated local IL-5 production by inducing Th1-mediated response [9]. Furthermore, recent studies suggested that mycobacteria induce not only Th cells providing Th1 type immune responses but also Treg cells. In an animal model of allergy, the immunomodulatory effects of *M. vaccae* was found to be mediated by allergen-specific regulatory T lymphocytes [37], and oral administration of *M. vaccae* inhibited pulmonary allergic inflammation by induction of IL-10 [14].

Alive BCG vaccination has been used for prevention of tuberculosis. The use of mycobacterium for immunomodulation requires repeated exposures to the immune system. However, repeated alive BCG vaccination is contraindicated. For human therapeutic application, it needs intradermal or intramuscular injection for vaccination. Unfortunately, cutaneous vaccination with mycobacterium species commonly produces granulomatous formation leading to recalcitrant ulcers. We need to develop Th1 type immunomodulating system that induces no granulomatous reaction, if species of mycobacteria are tried to use for human.The Ag85B protein is a main component of the cell wall of mycobacteria such as *M. tuberculosis and M. kansasii* [4]; this Ag85B is known as a strong Th1 inducer *in vitro* [17, 18]. Experiments using plasmid DNA encoding Ag85B has been previously reported. This Ag85B is able to protect against *M. tuberculosis* even in Balb/c mice [33]. Intraperitoneal administration of Ag85B DNA inhibits granulomatous changes or adhesive reaction of intraperitoneal organs in mice (data not shown). As a preliminary study, Ag85B DNA was intradermally injected in the skin of mice skin. No ulcerative changes were observed in vaccinated areas of the skin (data not shown).

In our present study, we evaluated the efficacy of DNA encoding Ag85B for inducing Th1 and Treg -type immune response in OX-induced acute phase dermatitis. Repeated applications of OX in mice ears caused Th2-type dominant dermatitis, which mimic most of the characteristic features of AD [16, 19, 21, 32]. We first investigated whether the application of Ag85B corrects the immune response from a type Th2 one to a type Th1 response. Our results showed that Ag85B successfully ameliorates Th2-cytokine dominant immediate type reaction in the skin lesions in both prophylactic and therapeutic models of the disease. In Ag85B-treated AD skin lesion, the ear swelling was significantly reduced compared to placebo-treated animals. Administration of Ag85B DNA suppressed histological abnormalities caused by atopic inflammations such as inflammatory cell infiltration, epidermal hyperplasia and severe edema. The presence of mast cells in the skin lesion is closely associated with Th2-type dermatitis; the number of mast cells was increased in OX-treated control animals as expected; however, the number of mast cells was decreased in Ag85B-treated mice compared with controls. Enhancement of the expression of IFN-γ mRNA was significant in Ag85B-treated AD mice compared with placebo-treated animals. The expression of IL-4 mRNA were suppressed in Ag85B-treated mice compared to placebo-treated controls (Fig. 4). In addition, serum IgE levels were significantly suppressed in Ag85B treated mice compared with placebo-treated mice. These finding demonstrates that administration of Ag85B DNA significantly inihibited the development of Th2-cytokine dominant skin atopic inflammation by inducing Th1-type immune response.

We also examined the potential of Ag85B to induce Treg cell responses. TGF-β and IL-10 have been described as critical regulatory cytokines produced by Treg [11]. Heat-killed *M. vaccae* induces regulatory T cells that secrete IL-10 and TGF-β [37]. *M. vaccae* also induces a population of $CD11^+$ cells characterized by an increased expression of regulatory cytokines including IL-10 and TGF-β [1]. Treg cells are developed mainly in the presence of IL-10 and TGF-β [13]. More recently, Inoue *et al* reported that topical application of CpG-Oligodeoxynucleotides induces $Foxp^+$ Treg in skin lesions of AD model mice in association with elevation of TGF-β [15]. Depletion of $CD4^+CD25^+$Treg from the peripheral blood of healthy individuals enhances proliferation of Th2 in response to various allergens [6, 23]. The mechanisms of the suppressive activity of Treg depend on cell-to-cell contact, and

there is evidence for the involvement of IL-10 and TGF- β [2, 3, 26]. In this study, we have shown elevated expression of TGF-β and IL-10 in Ag85B-treated mice (Fig.6a), and $Foxp3^+$ Treg was increased in the Ag85B-treated skin (Fig. 6b). We assume that the therapeutics capability of Ag85B is related to the induction of $Foxp3^+$Treg and Th1-type immune response.

In brief, in this study we have shown the usefulness of plasmid DNA of Ag85B for the amelioration of Th1/Th2 imbalance and for the generation of Treg cells. The observations suggest that Ag85B may be useful for the prevention and treatment of atopic disorders.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Grants-in-Aid for Core Research Evolutional Science and Technology.

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Figure legends

Fig. 1. Model of chronic contact hypersensitivity, and treatment with Ag85B DNA.

Fig. 2 (a). OX-induced ear swelling. The ear swelling response was expressed as the difference between ear thickness before and 30 min after each application on day 32. The columns and error bars represent mean ± SEM * $P<0.05$. Swelling was suppressed significantly in Ag85B-treated mice compared with those in placebo-treated mice. (b) Clinical features of ear skin on day 35. The OX-challenged mice showed severe skin eruption, however administration of the Ag85B DNA in both prophylactic and therapeutic models clearly reduced atopic inflammatory reactions in OX-sensitized mice.

Fig. 3. Histopathological features of skin lesions. Skin was taken on day 35, paraffin embedded sections were stained with hematoxylin & eosin (a), truidine blue (b). OX-challenged mice showed epidermal hyperplasia along with strong intra-dermal inflammatory cell infiltration; whereas Ag85B DNA significantly reduced the inflammatory changes.

Fig. 4. mRNA expression in the ear on day 35. In order to clarify the expression of cytokine mRNA, quantitative PCR was performed by using specific primers and probes for IL-4 and IFN-γ. The expression of IL-4 mRNA was reduced in Ag85B-treated mice compared with placebo-treated mice. On the other hand, mRNA expression of IFN-γ was significantly increased in Ag85B mice.

Fig. 5. Serum IgE concentrations. Serum IgE levels were measured on day35 in control, Ag85B DNA IP (-14, -7), or Ag85B DNA IP (14, 21) mice. The columns and error bars represent mean ± SEM. * $P<0.05$. Administration of Ag85B reduced IgE level.

Fig. 6 (a) mRNA expression in the ear on day 35. Quantitative PCR was performed by using specific primers and probes for IL-10 and TGF-β. Both TGF-β and IL-10 were increased in the Ag85B-treated mice. (b) Foxp3+ cells were clearly observed with confocal microscopy. (c) The number of $Foxp3^+$ cells per HPF was counted in five

Figure 1.

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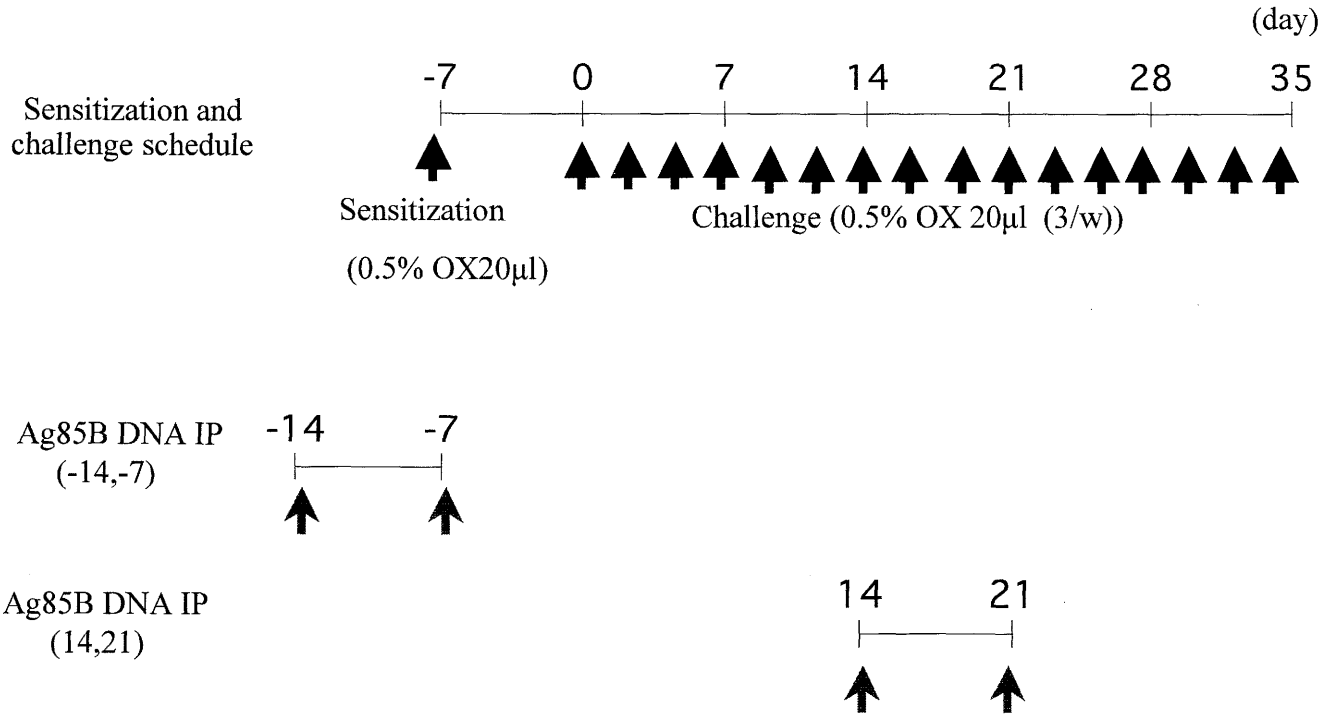

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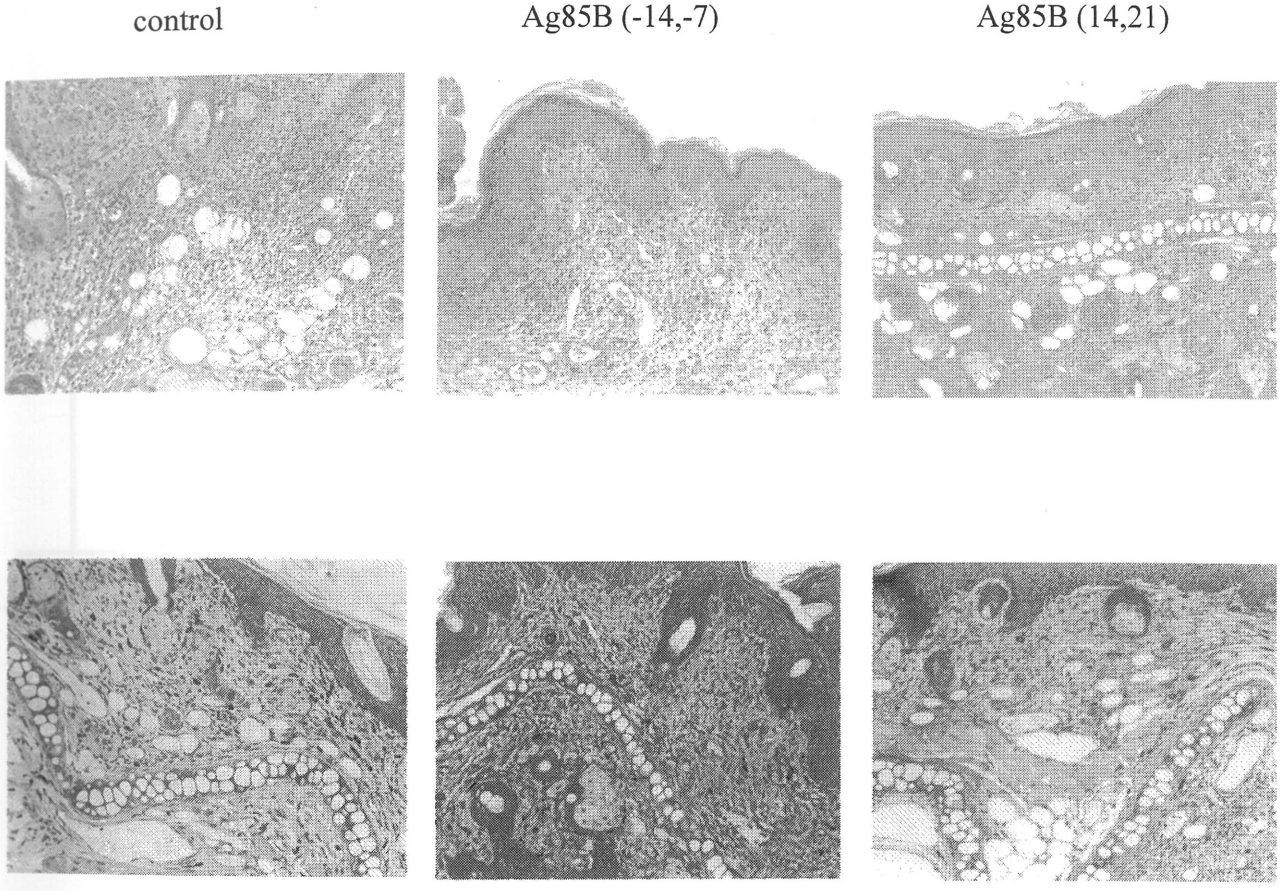

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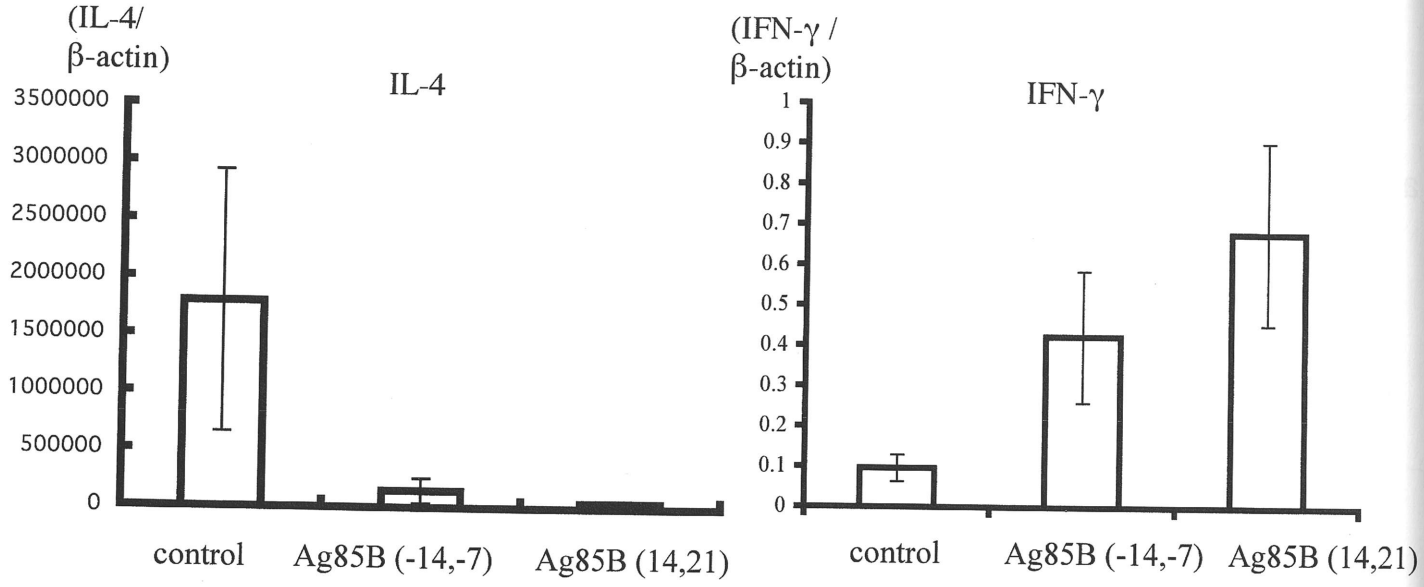

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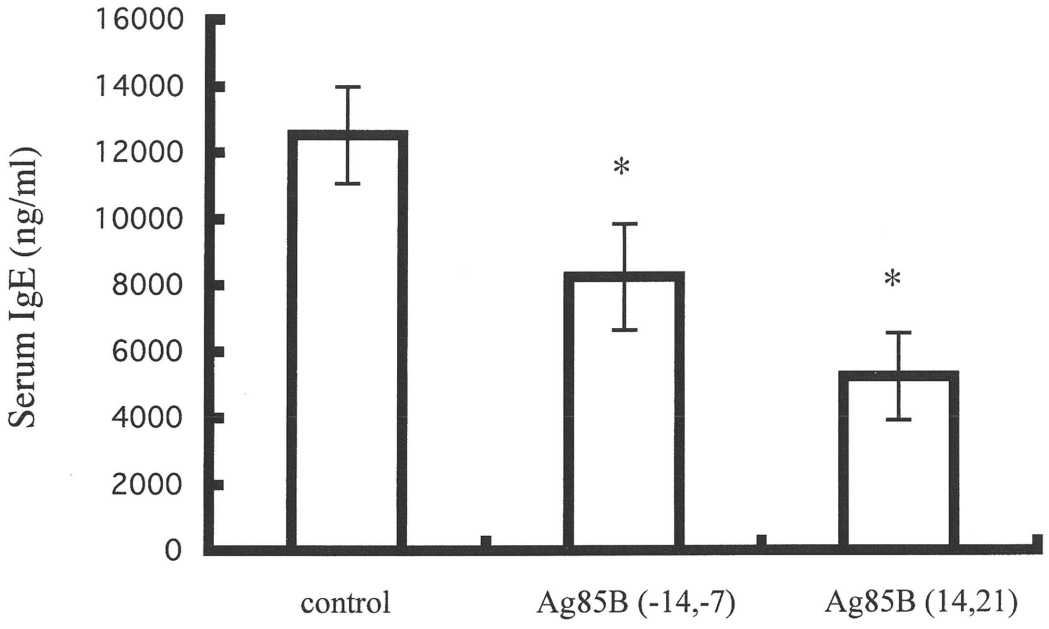

Figure 6.

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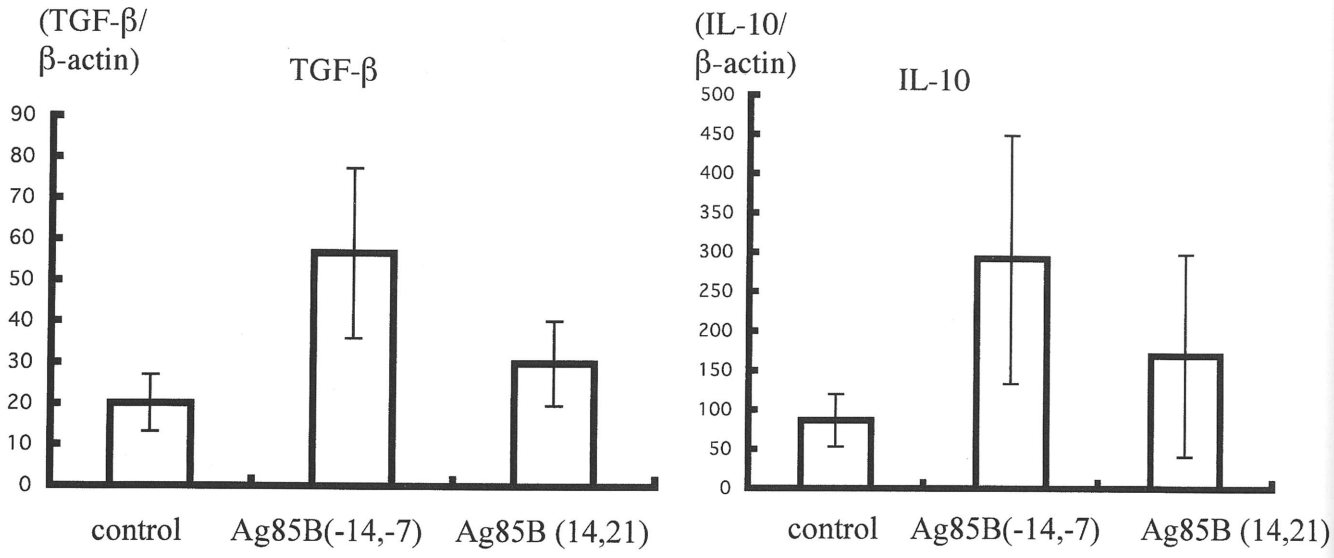
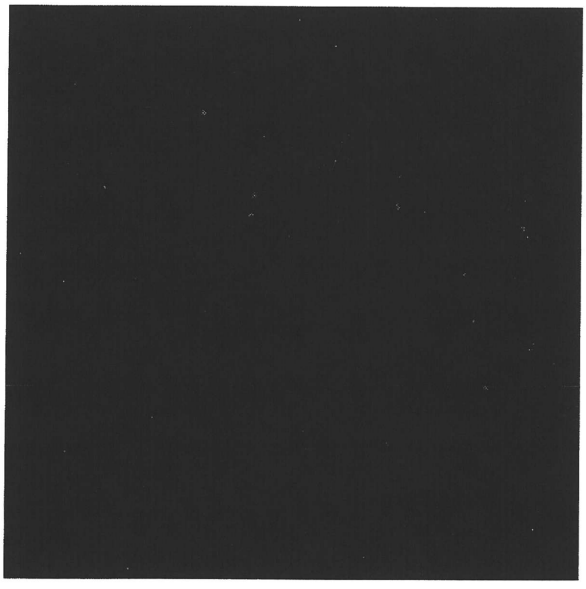
a



b



c