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Prevention of Macrophage-Related Inflammatory Diseases by Allergina

Sang B. Han[1], Chang W. Lee[1], Song K. Park, Won K. Yoon, Jae S. Moon, Ki H. Lee, Hyung C. Kim, and Hwan M. Kim[2]

Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Oundong, Yusong, Taejon 305-333, Korea

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The oriental herbal combination allergina has been shown to inhibit allergic inflammation. In the present study, we demonstrate that the oral administration of allergina markedly inhibits the progression of inflammatory diseases, such as graft-versus-host diseases (in the allogeneic bone marrow transplantation and the parent-into-F1 transplantation models), collagen-induced arthritis and sheep red blood cell-induced delayed type hypersensitivity. The immunosuppressive activity of allergina *in vivo* appears to be associated, at least in part, with the inhibition of tumor necrosis factor-α production. In conclusion, our results suggest that allergina could be useful as a immunosuppressive agent for the treatment of macrophage-related inflammatory disease.

Key words: Allergina, Graft-versus-host diseases, Collagen-induced arthritis, Delayed type hypersensitivity

INTRODUCTION

Allergina is a traditional medicinal formula, which consists of 16 herbal ingredients, and has been used for treatment of various allergic diseases in Korea. It was previously reported that allergina is very effective at inhibiting allergic inflammatory diseases, based on the discovery that this drug prevents compound 48/48-induced anaphylaxis and passive cutaneous anaphylaxis by inhibitng histamine release from mast cells (Jeong *et al.*, 2001). In addition, allergina showed clinically favorable outcomes when used as a treatment in pediatric patients having recurrent otitis media with effusions (Jeong *et al.*, 2002). Moreover, allergina was found to markedly decrease the production of macrophage-derived inflammatory cytokines, such as IL-1b, IL-6, IL-8 and TNF-α (Jeong *et al.*, 2001; Jeong *et al.*, 2002). In this context, we speculated that allergina might be effective at preventing other macrophage-related inflammatory diseases, such as graft-versus-host diseases, collagen induced arthritis and delayed type hypersensitivity, since it is known that the pathogenesis of these inflammatory diseases is associated with macrophages activation (Wagner *et al.*, 1988; Weisdorf *et al.*, 1990; Gongora *et al.*, 2000; Lu *et al.*, 2001; Luross and Williams, 2001; Gorgun *et al.*, 2002; Taylor *et al.*, 2002). In the present study, we investigated whether allergina is effective for the treatment of macrophage-dependent inflammatory disease.

Correspondence to: Hwan Mook Kim, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 52 Oundong, Yusong, Taejon 305-333, Korea
Tel: 82-42-860-4660, Fax: 82-42-860-4605
E-mail: hwanmook@kribb.re.kr

MATERIALS AND METHODS

Materials

Mice were purchased from Daehan Biolink Co. Ltd (Chungbuk, Korea) and maintained under SPF conditions until required. The KRIBB Animal Experimentation Ethics Committee approved the experimental procedures used in this study. Allergina was prepared from a prescription containing Schizonepetae herba, Forsythiae fructus, Ledebouriellae radix, Angelica radix, Cnidii rhizoma, Paeoniae radix alba, Angelicae dahuricae radix, Bupleuri radix, Auratinii fructus, Scutellariae radix, Fructus angelicae, Platycodi radix, Glycyrrhizae radix, Trichosanthis radix, Taraxaci herba, and Lonicerae flos, as described in the previous reports (Jeong *et al.*, 2001; Jeong *et al.*, 2002). A gram of allergina contained at least 4.6 mg and 4.5 mg of glycyrrhizic acid and hesperidine, respectively.

Graft-versus-host diseases (GVHD)

The effect of allergina on GVHD was determined in two different ways. To induce systemic GVHD in an acute lethal bone marrow transplantation model, recipient BALB/c mice (n=10) were administered 10 Gy of total body irradiation from a Soft M-150 WE (Softex, Tokyo, Japan) ^{60}Co source at a rate of 0.5 Gy/min (Lu *et al.*, 2001). One day after the irradiation, donor mice (C57BL/6 for allogeneic and BALB/c for syngeneic transplantation) were killed by cervical dislocation. Their femora were removed aseptically and bone marrow (BM) cells were prepared from the femoral shafts by inserting a 25-gauge needle at the proximal end. Splenic cells were prepared by passing softly ground spleens through a mesh. Recipient BALB/c mice (n=10) received a single injection of 0.25 mL of Phosphate Buffered Saline (PBS) containing 1×10^7 BM cells and 5×10^7 spleen cells through the tail vein. Allergina was orally administered to the recipient BALB/c mice once a day for 14 days from the day of transplantation.

To cause a local GVHD in the parent-into-F1 mouse model, spleen cells (5×10^6) isolated from parent C3H mice were subcutaneously injected into the right posterior footpad of recipient B6C3F1 mice (Okamoto et al., 2002). The same amount of spleen cells from B6C3F1 mice were injected into the opposite footpad of recipient B6C3F1 mice. On day 5, the bilateral popliteal lymph nodes (PLN) were removed and weighed. The intensity of local GVHD was expressed as the difference in the weights of the right and left PLN.

Collagen-induced arthritis (CIA)

Male DBA/1 mice were obtained from Charles River Japan Inc. (Yokohama, Japan). Bovine type II collagen was diluted in 0.05 M acetic acid to a concentration of 2 mg/ml, and emulsified in equal volumes of complete Freuds adjuvant (CFA, 2 mg/mL of Mycobacterium tuberculosis strain H37RA, Difco, Detroit, ML) (Han *et al.*, 2001). The mice (n=13) were immunized intradermally at the base of the tail with 100 μL of emulsion. On day 21, the animals were given an intraperitoneal booster injection of 100 μg of type II collagen dissolved in PBS. On day 28, the onset of arthritis was accelerated by a single intraperitoneal injection of 40 μg of lipopolysaccharide (LPS, Sigma, St. Louis). These mice were treated orally with allergina once a day from the day following the LPS injection for 4 weeks. Mice were examined visually for the appearance of arthritis in the joints and severity scores (macroscopic scores) were awarded as previously described (Han *et al.*, 2001). The clinical severity of arthritis was graded on a scale of 0-2 for each paw, according to changes in the redness and swelling, where 0=no change, 0.5=significant swelling and redness, 1.0=moderate, 1.5=marked, 2.0=maximal swelling and redness, and ankylosis.

Sheep red blood cell-induced delayed type hypersensitivity (sRBC-DTH)

SRBCs were reconstituted in PBS, washed three times with PBS by centrifugation and kept in the same medium. Mice were sensitized by the subcutaneous injection of 2×10^7 sRBC in 0.1 mL of PBS into the left hind footpad on day 0 (Gongora *et al.*, 2000). On day 4, sRBC-immunized mice were challenged by injecting 1×10^8 sRBC in 0.025 mL of PBS into the right hind footpad; the left hind footpad received solvent only. The thickness of the footpad was measured using a vernia caliper 24 h after challenge. The footpad reaction was expressed in mm as the difference in thickness between the right footpad injected with sRBC and the left with PBS. Allergina was administered orally at dose ranges from 100 to 1,000 mg/kg from day 0 to day 4.

In vivo TNF-α production

Mice were treated intravenously with 8 μg/0.2 mL of LPS (Conway *et al.*, 2001). Plasma was prepared from 90 min after LPS treatment and the TNF-α level was measured by ELISA, according to the manufacturers instruction (R&D systems, Minneapolis, MN). In this case, the allergina was orally administered 24 h and 2 h before the LPS injection.

RESULTS

Allergina prevents the progression of GVHD

The effect of allergina on GVHD was measured in two different ways. A systemic lethal acute GVHD was induced by the intravenous injection of allogeneic bone marrow and spleen cells from C57BL/6 mice into BALB/c mice. All recipient BALB/c mice without allergina treatment died within 8 days of transplantation (Fig. 1A). In these mice, clinical symptoms of acute GVHD, such as hair ruffling, lowered morbidity (data not shown) and weight loss (Fig. 1B), became apparent within 3 days. In contrast, the oral administration of allergina at doses of 500 or 1,000 mg/kg markedly reduced this mortality, and 70-80% of the mice survived up to 14 days (Fig. 1A). The protective effect of allergina on weight loss was not apparent until day 8, but body weight began to rise at day 9 and reached normal levels at day 13 (Fig. 1B). All syngeneic transplant mice survived for more than 14 days and underwent weight losses similarly to those of the allergina-treated mice (data not shown).

To cause a local GVHD, spleen cells from the parent C3H mice were subcutaneously injected into the right posterior footpad of the recipient B6C3F1 mice. The oral administration of allergina at 1,000 mg/kg significantly decreased the popliteal lymph node weight of the recipient mice (Fig. 2A). Allergina did not induce body weight loss,

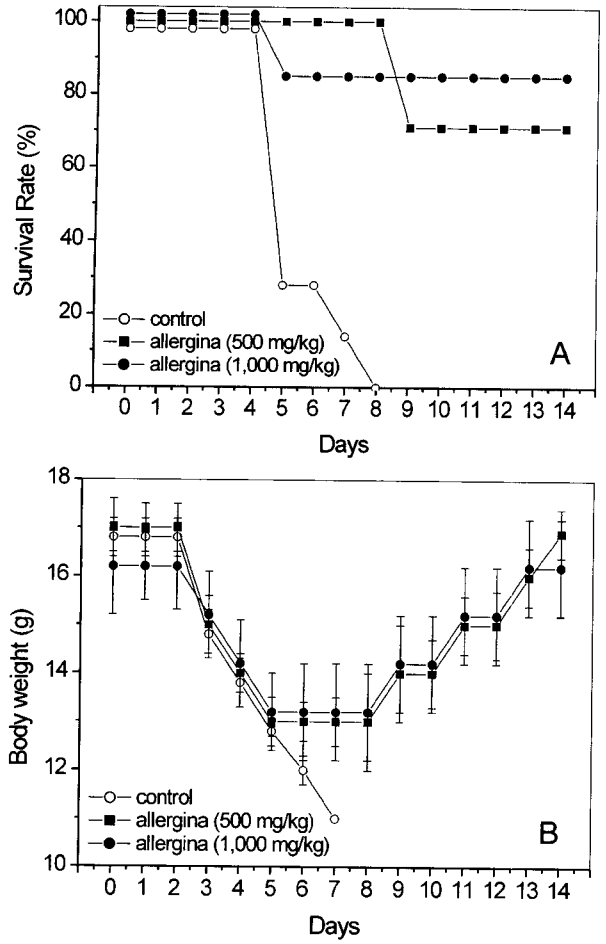

Fig. 1. The effect of allergina on systemic GVHD. Acute lethal GVHD was induced by intravenously injecting bone marrow and spleen cells from C57BL/6 mice into BALB/c mice (n=10) that had received 10Gy of total body irradiation, as described in *Materials and Methods*. BALB/c mice were treated daily with 500 or 1,000 mg/kg of allergina for 14 days. Control BALB/c mice were treated with distilled water; the medium used to dissolve the allergina. Survival rate (A) and body weights of the live mice (B) were measured daily.

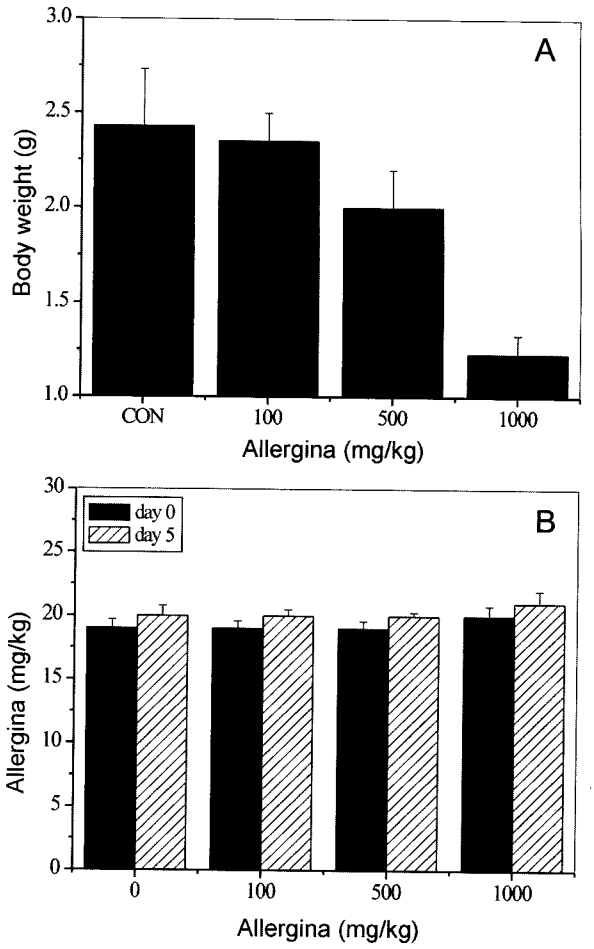

Fig. 2. The effect of allergina on local GVHD. Local GVHD was induced by the subcutaneous injection of spleen cells from parent C3H mice into the right footpad of B6C3F1 mice (n=5). The same number of spleen cells from B6C3F1 mice was injected into the opposite left footpad of the recipient mice. Allergina was orally administered at dose ranges from 100 to 1,000 mg/kg from day 0 to 4. Control mice were treated with distilled water, as used to dissolve the allergina. On day 5, the bilateral popliteal lymph nodes (PLN) were removed and weighed, and the intensity of the local GVHD was expressed as the difference the weights of the right and left PLN (A). The body weights of mice were also measured (B). Significance was determined using the Students t-test versus the control groups (*p<0.01).

and showed no appreciable side effects (Fig. 2B).

Allergina prevents the progression of CIA

DBA/1 mice were immunized with collagen/CFA on day 0, and further boosted with collagen on day 21 and LPS on day 28. As shown in Table I, the oral administration of 1,000 mg/kg of allergina for 4 weeks markedly delayed the pathogenesis of arthritis, based on the macroscopic arthritis score. All mice (100%) treated with allergina showed mild inflammation and clinical insulitis scores were less than 2.0 on the final day of allergina treatment. However, eighty-eight percent of the DBA/1 mice without allergina treatment showed severe joint inflammation and clinical insulitis scores exceeding 2.0.

Allergina prevents the induction of sRBC-DTH

To cause sRBC-DTH, CDF1 mice were sensitized with sRBC. Allergina was orally administered everyday at 100-1,000 mg/kg to see whether it has any effect on sRBC-DTH. As shown in Fig. 3A, the administration of allergina significantly decreased footpad thickness, showing a reduction of 48.0% at 1,000 mg/kg of allergina (Fig. 3A). Allergina did not induce body weight loss and cause any observable side effects (Fig. 3B).

Allergina prevents LPS-induced TNF-α production *in vivo*

Allergina at 100-1,000 mg/kg was administered orally

Table . Allergina delays the progression of collagen-induced arthritis

	Weeks	Clinical score[2]				
		0~1	1~2	2~4	4~6	6~8
Control	0	100[3]	–	–	–	–
	1	75	–	12	–	–
	2	12	63	12	–	–
	3	12	37	25	13	13
	4	0	12	62	13	13
Allergina[1]	0	100	–	–	–	–
	1	63	37	–	–	–
	2	37	63	–	–	–
	3	50	50	–	–	–
	4	0	100	–	–	–

[1]DBA/1 mice (n = 10) were orally administered with 1,000 mg/kg of allergina daily for 4 weeks, and control DBA/1 mice were treated with distilled water, which was used to dissolve allergina. [2]Mice were examined visually for the appearance of arthritis in the joints. The clinical severity of arthritis was graded on a scale of 0-2 for each paw, according to changes in redness and swelling, where 0 = no changes, 0.5 = significant swelling and redness, 1.0 = moderate, 1.5 = marked, 2.0 = maximal swelling and redness, and ankylosis. The allocated clinical score was a cumulative value for all paws (0-8). [3]The numerical value represents the percentage (%) to total mice (n = 10).

24 h and 2 h before LPS injection and plasma was prepared 90 min after LPS injection. The basal level of plasma TNF-α in chemically untreated mice was 10 pg/mL, and the plasma TNF-α levels increased to 307 pg/mL after LPS injection. The administration of 300 and 1,000 mg/kg of allergina significantly decreased this plasma TNF-α level by 185 and 100 pg/mL, respectively.

DISCUSSION

Acute GVHD is a major cause of morbidity and mortality in patients undergoing allogeneic bone marrow transplantation (BMT) (Wagner *et al.*, 1988; Weisdorf *et al.*, 1990; Gorgun *et al.*, 2002). Acute GVHD begins when donor type 1 helper T (Th1) cells recognize alloantigens presented by the hosts antigen presenting cells (APCs) via the T cell receptor/Major Histocompatibility Class (MHC) II interaction. Following this recognition, Th1 cells are further activated by the interaction of CD28/B7 and CD40/CD40L with APCs (Lu *et al.*, 2001; Taylor *et al.*, 2002). Collagen-induced arthritis (CIA) is the best-characterized animal model in terms of the pathogenesis and underlying immunological basis, and the histopathology is known to resemble human rheumatoid arthritis (Luross and Williams, 2001). In this regard, this model has been widely used for testing potential therapeutic agents for the treatment of human rheumatoid arthritis. Considerable evidence exists to suggest that the

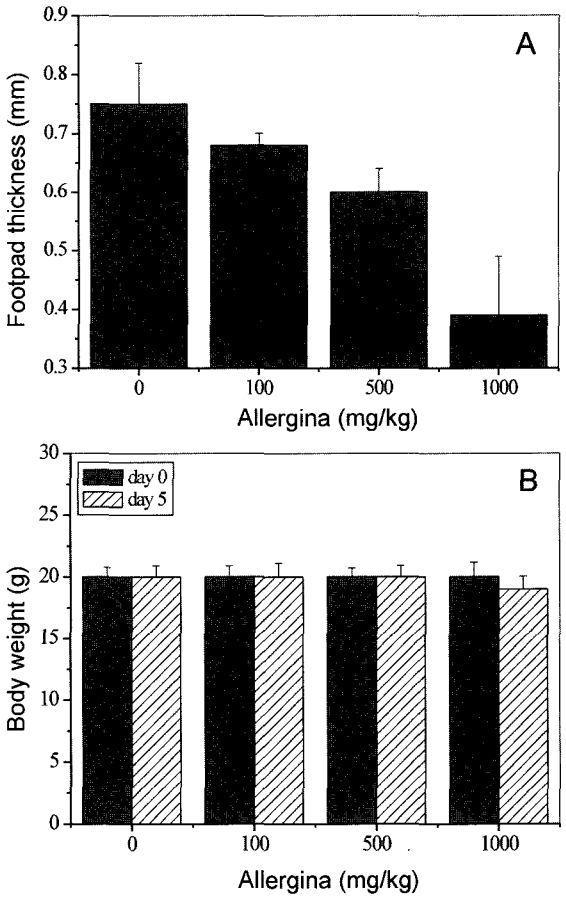

Fig. 3. The effect of allergina on sRBC-DTH. Mice were sensitized with sRBCs in the left hind footpad on day 0, and then challenged with sRBCs in the right hind footpad on day 4. Allergina was orally administered at 100-1,000 mg/kg from day 0 to day 4. On day 5, the thickness of the footpad was measured with a vernia caliper. The footpad reaction was expressed in mm as the difference in the thickness of the right footpad injected with sRBC and the left with PBS (A). Mice body weights were also measured (B). Significance was determined using the Students t-test versus the control groups (*p<0.01).

induction of CIA in DBA/1 mice is associated with Th1 cell activation. Delayed type hypersensitivity (DTH) is an expression of T cell-mediated immunity and plays a major role in the pathology of many inflammatory diseases (Gongora *et al.*, 2000). One classic form of the DTH inflammatory reaction mediated by MHC II-restricted CD4+ T cells is the one that caused by the subcutaneous injection of sRBCs. Overall, it could be concluded that the pathogenesis of these inflammatory diseases is associated with the activation of Th1 cells, which then produce IL-2 and IFN-γ. These cytokines activate macrophages to produce inflammatory effector molecules, especially TNF-α, causing tissue injury and disease. Ultimately, it is evident that macrophages play a pivotal role in the pathogenesis of GVHD, CIA and DTH.

In this context, we speculated that allergina might be

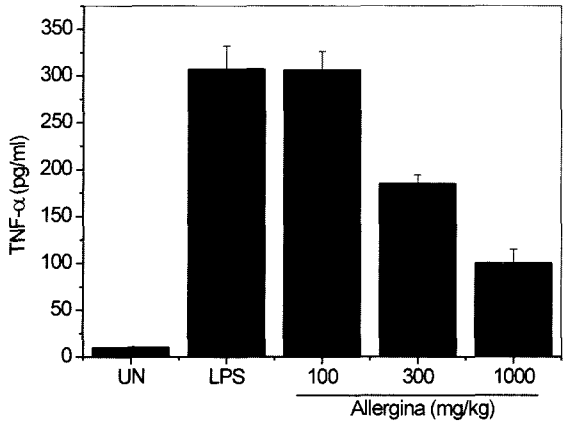

Fig. 4. The effect of allergina on LPS-induced TNF-α production in vivo. Mice were intravenously injected with 8 mg/0.2 mL of LPS. Plasma was prepared at 90 min after LPS treatment and the TNF-α level was measured by ELISA. Allergina (100-1,000 mg/kg) was administered 24 h and 2 h before the LPS injection. Significance was determined using the Students t-test versus chemically untreated control group (UN) (*p<0.01).

effective at preventing the progression of these inflammatory diseases, since allergina was known to inhibit macrophage-derived TNF-α production. In the present study, we also demonstrated that allergina significantly inhibits TNF-α production *in vivo*. Furthermore, we demonstrated that allergina appears to be very effective at preventing the disease progression of GVHD, DTH and CIA, which results, at least in part, from the inhibition of TNF-α production. Taken the results from the current study and the previous reports showing the effectiveness of allergina for treating allergy together, it is believed that allergina has a potential to be used as a therapeutic agent for the treatment of inflammatory diseases such as GVHD, CIA and DTH.

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