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Effects and mechanisms of aloperine on 2, 4-dinitrofluorobenzene-induced allergic contact dermatitis in BALB/c mice

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ABSTRACT

Allergic contact dermatitis is a prototypic T-cell-mediated cutaneous inflammatory response. Multiple cell types, inflammatory mediators and cytokines are involved in the regulation of immunologic and inflammatory processes in allergic contact dermatitis. Aloperine is an isolated alkaloid found in the plant of *Sophora alopecuroides* L. It has been clinically proved effective in China for a long time for skin inflammatory diseases such as allergic contact dermatitis. However, the mechanism of aloperine on allergic contact dermatitis is largely unknown. Therefore, the aim of this study was to investigate the effect of aloperine on 2, 4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis in BALB/c mice and the possible underlying mechanisms. The results showed that topical application of DNFB on the ear provoked typical allergic contact dermatitis with ear swelling and ear erythema in BALB/c mice. Treatments with 1% aloperine suppressed DNFB-induced increase in ear thickness and ear erythema. Moreover, 1% aloperine treatment significantly decreased the up-regulated mRNA and protein levels of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) induced by DNFB in ear biopsy homogenates. Our findings suggest that aloperine greatly improves the DNFB-induced allergic contact dermatitis in mice. The therapeutic mechanism might be related to the reduction of TNF-α, IL-1β and IL-6 production induced by DNFB.

1. Introduction

Allergic contact dermatitis, referred to as contact sensitivity, is a delayed-type hypersensitivity reaction mediated by hapten-specific T cells, which are primed in lymph nodes and recruited in the skin during the afferent and efferent phases of the reaction, respectively (Blauvelt et al., 2003). Bonneville et al. (2007) have demonstrated that allergic contact dermatitis to the strong hapten 2, 4-dinitrofluorobenzene (DNFB) in mice is mediated by $CD8^+$ cytotoxic T cells and down-regulated by $CD4^+$ T cells. Many studies have revealed that multiple cytokines and chemokines are also involved in the regulation of the process in allergic contact dermatitis (Gober and Gaspari, 2008; Kondo and Sauder, 1995). Among these, it is widely recognized that the pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) and inflammatory cytokines (e.g. IL-6, IL-8) play key roles in the pathogenesis of allergic contact dermatitis.

Allergic contact dermatitis is one of the most common dermatoses which is caused by hard-to-avoid common allergens such as nickel and rubber products. Its socioeconomic impact as an acquired, job-related disease is enormous (Enk, 1997). Allergic contact dermatitis affects approximately 7% of the population and the incidence of allergic contact dermatitis is steadily rising (Johansson et al., 2004; Schnuch et al., 2002). Most current topical therapies for allergic contact dermatitis include glucocorticosteroids, non-steroid anti-inflammatory drugs, and immunomodulators (Atarashi et al., 2008; De Vry et al., 2005; Letko et al., 1999; Reitamo et al., 2002; Wolff and Stuetz, 2004; Worm, 2002). However, these agents are often associated with severe adverse effects (Fisher, 1995; Ingber, 2002). Therefore, there is a great need for the development of new and effective therapies for allergic contact dermatitis.

Aloperine is an isolated alkaloid in sophora plants such as *Sophora alopecuroides* L, which has shown anti-inflammatory and anti-virus properties (Qavi et al., 2002; Zhou et al., 1989). Clinically, aloperine has been widely used in China for decades to treat patients with allergic contact dermatitis, eczema and other skin inflammation. The chemical structure of aloperine is shown as follows.

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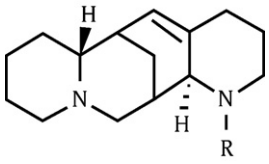

Chemical structure of aloperine (C15H24N2, MW = 214).

Although aloperine has shown anti-inflammatory properties against allergic contact dermatitis and other skin inflammatory diseases, the mechanism of aloperine on allergic contact dermatitis has not been elucidated yet. The aim of this study, therefore, is to evaluate the effects of aloperine on allergic contact dermatitis in BALB/c mice. We examined the effect of aloperine on DNFB-induced ear swelling and ear erythema. Protein and mRNA levels of cytokines TNF-α, IL-1β and IL-6 in DNFB-induced mice ear were also investigated. Our results suggest that the therapeutic effect of aloperine on chemical-induced allergic contact dermatitis might be related to its anti-inflammatory action.

2. Materials and methods

2.1. Animals

Female BALB/c mice (8–10 weeks old, 16–18 g body weight) were purchased from Weitonglihua Company (Beijing, China). All animals were healthy, housed in five mice per cage at 20 °C. Water and a standard diet (Weitonglihua Company, Beijing, China) were available *ad libitum*. All animal experiments were approved by the Animal Study committee of the Air force General Hospital according to government guidelines for animal care.

2.2. Induction of allergic contact dermatitis in BALB/c mice

Allergic contact dermatitis was induced in BALB/c mice according to a published method with minor modifications (Bhol and Schechter, 2005). Briefly, DNFB (presented by Dr. Lu, Peking University, Beijing, China) was dissolved in AOO (acetone:olive oil = 4:1) and used as an inducer of allergic contact dermatitis. The hair on abdominal skin was shaved. For active sensitization, 100 μl 0.5% DNFB was topically applied to the shaved abdomen. Five days later, the mice were challenged by painting the inner and outer surfaces of both sides of the ears with 20 μl 0.2% DNFB. The animals were rechallenged after one day to yield extensive disease.

2.3. Groups and treatment

0.1% momestasone furoate (Shenyang Pharmaceutical Company, Shenyang, China), high potent steroid, was used as a positive control. Aloperine was purchased from Chinese State Department of Drug Detection (Beijing, China) and mixed in the emollient cream vehicle for 0.1% momestasone furoate 0.1% (W/W). Aloperine cream was applied to both ears at 0.5 h, 24.5 h, 48.5 h and 72.5 h after rechallenge, respectively. The experiment mice were randomly divided into four groups ($n = 8$) as follows: control group (normal mice without any treatment), DNFB group (DNFB-rechallenge plus no drug treatment), DNFB + momestasone furoate group (DNFB-rechallenge plus 0.1% momestasone furoate treatment), DNFB + aloperine group (DNFB-rechallenge plus 1% aloperine treatment).

2.4. Ear swelling and ear erythema score

The extent of ear swelling and erythema were used as a measure of allergic contact dermatitis. Ear thickness was measured before challenge and then pre- and post-treatment (0, 24 h, 48 h, 72 h, 96 h after rechallenge respectively) using a dial thickness gauge (Mitutoyo Corporation, Kanakawa, Japan). Erythema was scored on a scale of 0–4 as previously described (Bhol and Schechter, 2005). Briefly, 0 was absent and 4 was severe pinkish red color of the ear.

2.5. Histopathology

96 h after rechallenge, the mice were killed by CO_2 asphyxiation. Then 5 mm of the ears was punched and fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. After the skin sections were stained with hematoxylin and eosin, the microscopic examination was carried out and examined by a board certified veterinary pathologist unaware of the treatment of the samples being evaluated.

2.6. Analysis of cytokine production in inflammatory regions

Cytokines protein levels were determined by enzyme-lined immunosorbent assay (ELISA). Ear samples ($n = 8$) of the inflamed region were weighed (100 mg) and homogenized in 1 ml of T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail. Homogenates were then centrifuged at 12,000 × g for 20 min at 4 °C to obtain the supernatant. TNF-α, IL-1β and IL-6 levels were analyzed using Biotrak ELISA system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Cytokine levels were normalized with a protein assay kit and calculated as pg per mg total protein.

2.7. Quantification of mRNA by semi-quantitative RT-PCR

Specific mRNA levels were determined by semi-quantitative RT-PCR using 18S rRNA as an internal control (all chemicals were from Invitrogen). The details of all oligonucleotide primer sequences are listed in Table 1. Total RNA was extracted and quantified as previously reported (Cruz et al., 2008). Briefly, 1 μg of RNA was incubated with 250 ng of random primers, and 200 U of reverse transcriptase (SuperScript III) for 30 min at 50 °C. A 1:10 dilution of cDNA was subjected to PCR amplification using 20 pmol of primers and 0.5 U of Platinum Taq DNA polymerase. The cycling program was: 3 min incubation at 94 °C, 12–47 cycles of amplification, denaturation for 50 s at 94 °C, primer annealing for 40 s, extension for 40 s at 72 °C, and 7 min extension at 72 °C. 10 ml of the PCR products was separated by electrophoresis on a 2% agarose gel. Gels were stained with ethidium bromide, and PCR products were quantified by densitometry with Image Analysis Software DigiMic800 (developed by the Air Force General Hospital and Bei Hang University together).

2.8. Statistical analysis

Data are presented as mean ± S.D. Comparison of more than two groups was made with a one-way analysis of variance ANOVA followed by Dunnett *t* test. A *P* value less than 0.05 was considered significant.

Table 1
Primer sequences used in this study.

Gene name	Primer sequence (5′–3′)
TNF-α	Forward: AGTCCCCAAACAACCTCCAT
	Reverse: TTGACCGCTGAAGAGAACCT
IL-1β	Forward: TGGCACGTATGAGCTGAAAG
	Reverse: CAGGAAGACGGGCATGTACT
IL-6	Forward: ACCTGCCTGCTGAGAATCACT
	Reverse: TTGGCTCTGTAACAGGGGATAT
18S rRNA product	Forward: GGACAGGATTGACAGATTGATAG
	Reverse: CTCGTTCGTTATCGGAATTAAC

3. Results

3.1. Effects of topical application of aloperine on DNFB-induced allergic contact dermatitis in BALB/c mice

Topical application of 1% aloperine significantly reduced the DNFB-induced ear thickness at 72 h and 96 h post-treatment (both $P<0.05$), as shown in Fig. 1A. Momestasone furoate treatment also significantly decreased the DNFB-induced ear thickness at 72 h and 96 h post-treatment ($P<0.05$ and $P<0.01$, respectively). Similar results were observed in the DNFB-induced ear erythema (Fig. 1B). The effect of aloperine and therapeutic treatment of momestasone furoate was not significantly different. Histopathological analysis also demonstrated that DNFB-induced increase in ear thickness was suppressed by aloperine treatment (Fig. 2). We can also observe the inhibition of DNFB-induced leukocytes infiltration into epidermis and dermis by aloperine and momestasone furoate treatment.

3.2. Effects of topical application of aloperine on cytokines protein expression in inflammatory regions determined by ELISA

An important mechanism for allergic contact dermatitis is the secretion of cytokines such as TNF-α, IL-1β and IL-6 (Gober and Gaspari, 2008; Kondo et al., 1995). Protein levels of cytokines TNF-α, IL-1β and IL-6 were measured in DNFB-induced allergic contact dermatitis. As shown in Fig. 3, DNFB challenge resulted in an increase in TNF-α, IL-1β and IL-6 protein levels in ear biopsy homogenates (all $P<0.01$). Compared with DNFB group, 1% aloperine treatment significantly reduced protein levels of TNF-α, IL-1β and IL-6 cytokines ($P<0.05$, $P<0.05$, $P<0.01$, respectively). Thus, topical application of 1% aloperine may reduce local secretion of cytokines, thereby reducing cutaneous allergic contact dermatitis induced by DNFB. Treatment with 0.1% momestasone furoate also significantly decreased the DNFB-induced increase in TNF-α, IL-1β and IL-6 cytokine production (all $P<0.01$).

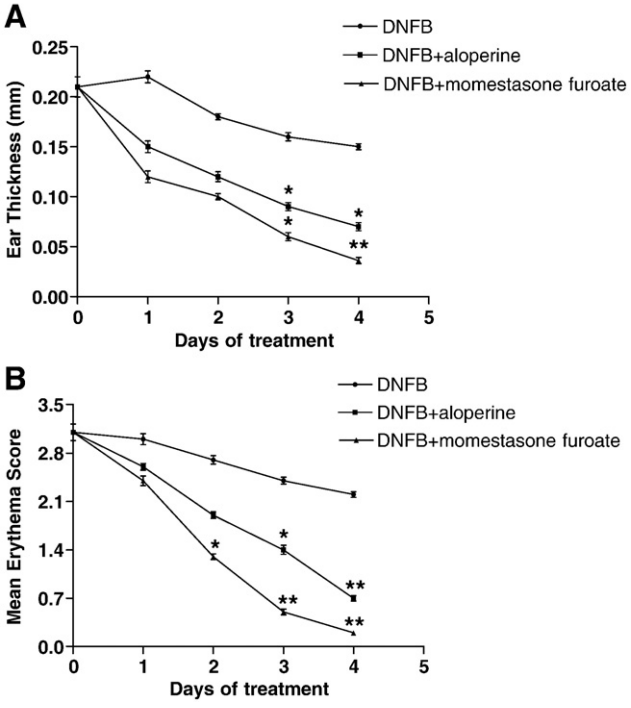

Fig. 1. Effect of 1% aloperine on DNFB-induced ear swelling and erythema in BALB/c mice. (A) Ear thickness scores as a function of treatment in mice with allergic contact dermatitis. (B) Erythema scores as a function of treatment in mice with allergic contact dermatitis. Data are presented as mean ± S.D. ($n=8$). *$P<0.05$, **$P<0.01$, as compared to the DNFB group.

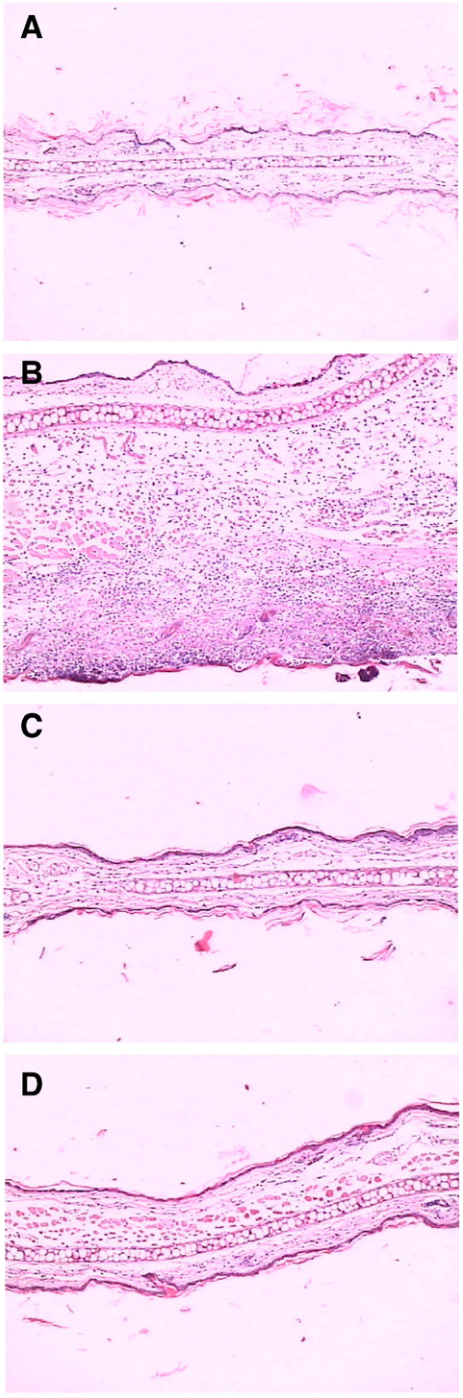

Fig. 2. Histopathological analysis of 1% aloperine on DNFB-induced allergic contact dermatitis in BALB/c mice. Untreated control ear (A), DNFB-treated ear (B), DNFB + momestasone furoate treated ear (C), and DNFB + aloperine treated ear (D) were displayed. Sections were stained with hematoxylin–eosin, micrographies at 100×. Sections shown are representatives of more than five observations.

3.3. Effects of topical application of aloperine on cytokines mRNA expression in inflammatory regions determined by semi-quantitative RT-PCR

To get better insights into the molecular mechanisms involved in the development of allergic contact dermatitis reactions we next analyzed

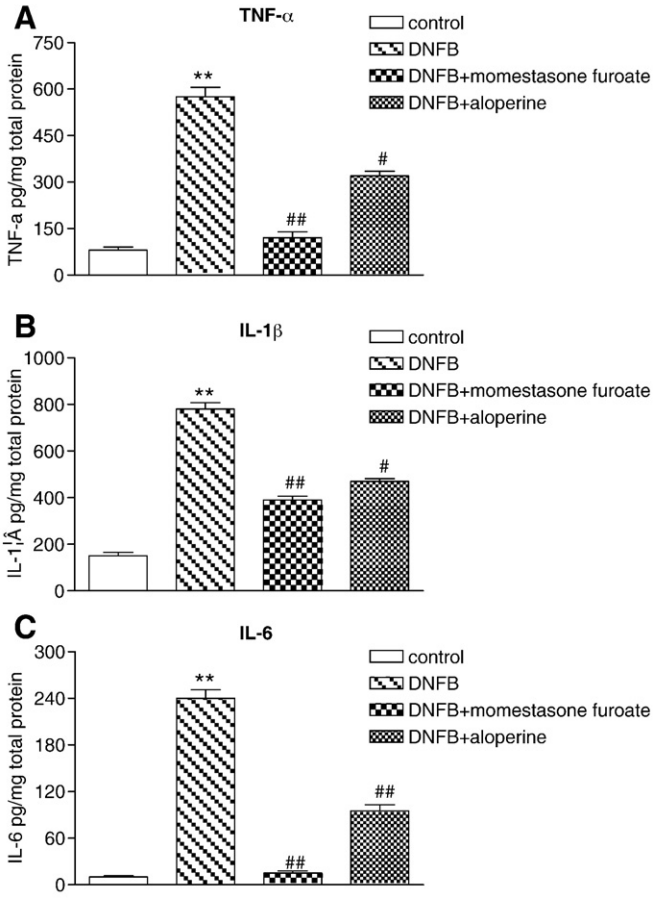

Fig. 3. Effect of aloperine on DNFB-induced expression of TNF-α (A), IL-1β (B) and IL-6 (C) protein levels. Ear punch biopsies were taken 24 h following the last treatment, and tissue homogenates were examined for cytokine production using ELISA. Cytokine levels were calculated as pg per mg total protein. $^{**}P<0.01$, as compared to the control group; $\#P<0.05$, $\#\#P<0.01$, as compared to the DNFB group.

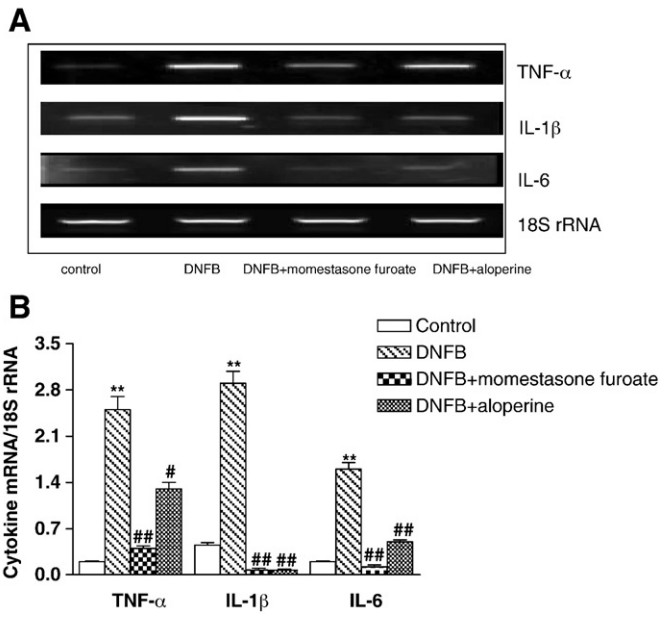

Fig. 4. Effects of aloperine treatment on the mRNA levels of TNF-α, IL-1 β and IL-6 in ear tissues of mice. (A) Representative electrophoretic analysis of RT-PCR products. (B) Relative ratio of densitometry of TNF-α, IL-1β and IL-6 to 18S rRNA. $^{**}P<0.01$, as compared to the control group; $\#P<0.05$, $\#\#P<0.01$, as compared to the DNFB group.

the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β) and inflammatory cytokine (IL-6) in the ear skin by semi-quantitative RT-PCR. As shown in Fig. 4, the ear skin of untreated BALB mice contained only trace amounts of TNF-α, IL-1β, and IL-6 messenger RNA (mRNA). DNFB induced a significant up-regulation of mRNA levels of TNF-α, IL-1β, and IL-6 (all $P<0.01$). Topical application of 1% aloperine significantly decreased the up-regulation of these cytokines, as compared to DNFB-treated mice ($P<0.05$, $P<0.01$, $P<0.01$, respectively).Treatment with 0.1% momestasone furoate also effectively down-regulated the mRNA expression of these cytokines to near normal levels (all $P<0.01$).

4. Discussion

Allergic contact dermatitis is the most common job-related disease of the western world. Often, the sensitizing allergens are common and difficult to avoid. However, the limitations of current drugs (e.g. corticosteroids) are becoming increasingly evident. Better treatment from the perspectives of efficacy and side effects are needed. Aloperine is one of the major alkaloids found mostly in the seeds of *S. alopecuroides* L, which is a kind of wild plant living in very cold and arid environment. And now *S. alopecuroides* L has been listed as one of protected Chinese natural herbal medicine resources in China. Aloperine has been used in China for decades to treat patients with inflammatory skin diseases such as allergic contact dermatitis. However, the underlying mechanism is still inconclusive. Therefore, we hypothesized that the mechanism of aloperine on allergic contact dermatitis is by inhibiting certain inflammatory pathways. In the present study, we demonstrated that topical application of aloperine suppresses DNFB-induced ear swelling in mouse model of allergic contact dermatitis. To the best of our knowledge, this is the first report showing the inhibitory effect of aloperine on chemical-induced allergic contact dermatitis in mice. The effect of aloperine on allergic contact dermatitis was further supported by histopathological analysis showing that a DNFB-induced increase in ear thickness and infiltration of leukocytes into epidermis and dermis was suppressed by aloperine treatment.

Topical applications of corticosteroids are one of the most frequently prescribed medications for treatment of various cutaneous inflammatory diseases such as allergic contact dermatitis and other skin inflammatory diseases, although the use of corticosteroids needs to be regulated due to potential adverse effects (e.g. skin atrophy, hyperpigmentation). Therefore, we compared the effect of aloperine with that of 0.1% momestasone furoate on allergic contact dermatitis in the present model. We found that the efficacy of 1% aloperine was comparable to that of 0.1% of momestasone furoate on the DNFB-induced allergic contact dermatitis. As an alkaloid isolated from traditional Chinese medicine, aloperine has few side effects in treating allergic contact dermatitis in clinical practice. The present results showed that aloperine could replace corticosteroids for treatment of allergic contact dermatitis.

Allergic contact dermatitis results from a T-cell response to harmless, low-molecular weight chemicals (haptens) applied to the skin (Inagaki et al., 2006). Haptens are small, chemically reactive substances that are only recognized by the immune system when bound to a protein or a peptide structure. Because these haptens are very chemically reactive, they bind rather nonspecifically to a multitude of structures when applied to the skin. However, binding to epidermal Langerhans cells (LCs) is considered to be the critical interaction (Yamashita et al., 2009). Studies have shown that the number of LCs present in the skin directly correlates with the ease with which the animal can be sensitized to an allergen. Although the exact mechanism of LCs activation remains unclear, recent studies have shown that tyrosine kinases of the Src family, mitogen-activated protein kinase (MAPK) family, the nuclear factor kappa B (NF-κB) and Keap1/Nrf2 pathways are involved in the early signal transduction

events (Ade et al., 2007; Ade et al., 2009; Cruz et al., 2004; Enk, 1997; Koeper et al., 2007; Trompezinski et al., 2008). Only 15 min after the binding of the allergen to the LC, the cell starts to up-regulate IL-1β mRNA and protein production (Enk and Katz, 1992). IL-1β is a primary cytokine that can induce other pro-inflammatory cytokines. By releasing IL-1β, the LC therefore induces other epidermal cells such as keratinocytes to become activated and produce other cytokines. The release of IL-1β by LC seems to be essential for this process, because neutralization of IL-1β with a specific antibody prevents the induction of all other cytokines and also epicutaneous sensitization. The production of IL-1β by LCs induces a cascade of various other cytokines, mostly from keratinocytes. Among them are TNF-α and IL-6. This mixture of cytokines enhances the maturation status of LCs. As a result, LCs up-regulate major histocompatibility complex (MHC) class II molecules, adhesion molecules and co-stimulatory factors, and mature from potent antigen-processing cells into potent antigen-presenting cells, which in term resulting in allergic contact dermatitis. Therefore, TNF-α and IL-1β are key mediators of the cutaneous inflammatory response (Kock et al., 1990; Larrick et al., 1989; Piguet et al., 1991). Previous reports suggested that the inhibition of pro-inflammatory TNF-α and IL-1β might be beneficial for the treatment of allergic contact dermatitis (Griffiths et al., 2005; Groves et al., 1995; Olmos et al., 2007; Watanabe et al., 2007). In the present study, we also focused on the effect of aloperine on suppression of inflammatory cytokines by measuring the mRNA and protein expression of TNF-α and IL-1β. Consistent with the previous study, we found that mRNA and protein levels of TNF-α and IL-1β were up-regulated in the DNFB-induced allergic contact dermatitis mice. 1% aloperine treatment significantly reduced the DNFB-induced increases on mRNA and protein levels of pro-inflammatory cytokines TNF-α and IL-1β.

IL-6 is a major mediator of the acute phase response of allergic contact dermatitis, because it enhances the production of acute phase proteins (Heinrich et al., 1990). The important role of IL-6 in inflammation is also supported by the research showing increased IL-6 plasma levels after elicitation of allergic contact dermatitis in mice (Kimber et al., 1990). Another study showed an increase in keratinocyte-bound IL-6 in allergic patch test reaction sites. IL-6 also plays a role in the local control of LC number and function (Oxholm et al., 1991). IL-6, like IL-1, is also able to function as a second signal required for thymocyte and T-cell proliferation (Kondo and Sauder, 1995). We also analyzed the expression of IL-6 in the ear of DNFB challenged mice. As expected, the mRNA expression of IL-6 was increased by DNFB treatment and this was suppressed by 1% aloperine treatment.

Our preliminary study showed that mice challenged once with DNFB did not induce significantly ear swelling and ear erythema compared with control mice. However, 1% aloperine treatment could also suppress these milder inflammatory skin diseases (data not shown). Therefore, in the present study, the model for allergic contact dermatitis has been modified as previously described (Bhol and Schechter, 2005). The BALB/c mice were challenged twice to yield extensive disease, such as ear swelling, ear thickness and infiltration of leukocytes into epidermis and dermis. This elicitation model might be more reasonable to investigate the effects of aloperine on allergic contact dermatitis.

Studies have indicated a crucial role for Th17 cells in eliciting murine allergic contact dermatitis (He et al., 2006). A recent study showed that Th17 cells mediated inflammation plays a pivotal role in the immunopathology of allergic contact dermatitis (Larsen et al., 2009). However, it remains unclear whether aloperine down-regulates the inflammatory cytokine production of IL-1β, TNF-α and IL-6 from reducing Th17 cells. This is an interesting question, which needs further analysis. Furthermore, as allergic contact dermatitis is a prototypic T-cell-mediated disease, future studies are needed to study the effect of aloperine on regulatory T cells in DNFB-induced allergic contact dermatitis. These data will strengthen our results and give details on mode of action.

Collectively, our results showed that the inhibitory effect of aloperine on allergic contact dermatitis might be mediated, at least in part, by blocking the mRNA and protein levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and subsequent down-regulation of IL-6 expression. The present data suggested that aloperine modulates these cytokine expression at both transcription and post-transcription levels in DNFB-induced allergic contact dermatitis. Our results also suggest that aloperine can serve as a potential therapeutic agent for prevention and treatment of cutaneous inflammatory diseases.

Acknowledgments

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