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Possible inhibitory mechanism of FK506 (tacrolimus hydrate) ointment for atopic dermatitis based on animal models

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Abstract

The effects of FK506 (tacrolimus hydrate) ointment on cutaneous allergic reactions in mice and rats were investigated. FK506 ointment showed significant suppressive effects on delayed allergic reactions in both species, and especially in rats, its inhibitory action was much stronger than that of alclometasone dipropionate, a so-called medium class steroid ointment. On the other hand, FK506 ointment did not inhibit the immediate allergic reaction in rats. FK506 ointment suppressed the delayed allergic reactions in locally passively sensitized mice to the same degree as that in actively sensitized mice. Accordingly, it is speculated that FK506 ointment inhibits the activation of sensitized T lymphocytes (Th1 cells) already accumulated in the dermis.

Keywords: Atopic dermatitis; FK506 ointment; Animal model; Delayed allergic reaction; Inhibitory mechanism

1. Introduction

Atopic dermatitis is a chronic allergic inflammatory disease which manifests itself as eczematous skin lesions (Mihm et al., 1976). Recently, in increasing numbers of patients, particularly in adults, the growing severity of lesions and resistance to the steroid therapy have been serious problems. Although the precise mechanism underlying atopic dermatitis has remained unclear, it appears that immediate (Immunoglobulin E (IgE)-mediated mast cell type), late (IgE-mediated Th2 type) and delayed (IgE-independent Th1 type) allergic reactions are involved in atopic dermatitis (Kapsenberg et al., 1991; Grewe et al., 1994, 1995; Hamid et al., 1994; Yamada et al., 1995; Thepen et al., 1996). Especially Grewe's and Thepen's groups have suggested that there is a biphasic pattern in atopic dermatitis, starting with a Th2 type allergic reaction, being allergen-specific, followed by a Th1 type allergic reaction, and that the Th2 type is important at the induction of inflammation, whereas the Th1 type is responsible for the maintenance and aggravation of the inflammation representing the chronic phase of atopic dermatitis. A drug which suppresses both types of allergic reactions would thus be useful for the treatment of atopic dermatitis.

FK506 (generic name: tacrolimus hydrate) is a novel macrolide immunosuppressant discovered in 1984 by the Exploratory Research Laboratories of Fujisawa Pharmaceutical (Kino et al., 1987), and is now used for liver and kidney transplantation worldwide. FK506 ointment has been developed as a topical treatment and showed good efficacy in clinical studies for atopic dermatitis (Nakagawa et al., 1994; Aoyama et al., 1995; Ruzicka et al., 1997). The results in the Phase III clinical studies of FK506 ointment in Japan have clearly demonstrated that it is superior to alclometasone dipropionate ointment, the medium class of steroid ointments and equal to betamethasone valerate ointment, the strong class of steroid ointments, in efficacy. We have reported that FK506 ointments showed suppressive effects on several animal models of atopic dermatitis, for example, the spontaneous dermatitis model in NC/Nga mice (Hiroi et al., 1998), the skin inflammation model using repeated topical application of antigens in rats (Fujii et al., 1997) and late and delayed cutaneous allergic reaction models in mice (Sengoku et al., 1998). Also, topical FK506 solution was reported to be effective in contact allergic reactions of domestic pigs, guinea pigs and the human (Lauerma et al., 1992, 1994; Meingassner, 1992; Lauerma and Maibach, 1994). Based on the results of these studies, the main inhibitory mecha-

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nism of FK506 ointment was thought to be inhibition of Th2 and Th1 type allergic reactions.

The effects of FK506 ointment on immediate and Th1 type allergic reaction were now further investigated to confirm the inhibitory mechanism of FK506 ointment for atopic dermatitis in rats and mice. Although the Th1 type allergic reaction in atopic dermatitis is probably allergen-non-specific, we used the tuberculin-induced delayed allergic reaction model, which is being antigen-specific, because it was a typical and simple Th1 type reaction.

2. Materials and methods

2.1. Animals and materials

The experiments were approved by the Fujisawa Pharmaceutical Animal Experiment Committee and were carried out following the guidelines for animal experiments at Fujisawa Pharmaceutical.

Female BALB/c mice (7-weeks-old), female C3H/He mice (7-weeks-old), male C57BL/6 mice (7-weeks-old), male Lewis rats (7-weeks-old) and male Brown–Norway rats (8-weeks-old) were obtained from Nippon Charles River (Hino, Japan). They were allowed to adapt to the environment for at least 4 days and were maintained on a standard diet with water ad libitum.

Mycobacterium tuberculosis, Freund's incomplete adjuvant, Freund's complete adjuvant and purified protein derivative (PPD) were purchased from Difco (Detroit, MI, USA), Difco, Wako (Osaka, Japan) and Nippon-BCG (Tokyo, Japan), respectively. Egg albumin and Evans blue were purchased from Seikagaku Kogyo (Tokyo, Japan) and Nakalai Tesk (Osaka, Japan), respectively. Antiserum of Sprague–Dawley rats containing anti-Egg albumin IgE (Sprague–Dawley rat passive cutaneous anaphylaxis titer 1:64), and 0% (base), 0.3% and 1% of FK506 ointments were prepared in Fujisawa Pharmaceutical (Osaka, Japan). Alclometasone dipropionate ointment (almeta® ointment) (0.1%) was purchased from Shionogi (Osaka, Japan). Dial thickness gauge and spectrometer were purchased from Ozaki (Tokyo, Japan) and Molecular Devices (Sunnyvale, USA), respectively.

2.2. Tuberculin-induced delayed allergic reaction in actively sensitized mice

A suspension of killed *M. tuberculosis* in saline (5 mg/ml) was emulsified with an equal volume of Freund's incomplete adjuvant, and 200 μl of the emulsion was divided into four parts and injected subcutaneously in mice for sensitization. After 2 weeks, tuberculin-induced delayed allergic reaction was elicited by intradermal injection of PPD (5 μg) into the inner side of both ears in a volume of 10 μl. The delayed allergic reaction was assessed quantitatively by measuring the ear thickness and amount of dye leaked in the ears. In detail, at 24 h after the challenge, the ear thickness was measured with a dial thickness gauge, and then, mice injected intravenously with 0.5% Evans blue 4 h previously in a volume of 0.25 ml were killed and the amount of dye leaked in both ears was determined using a spectrometer. Mice were anesthetized with ether during the sensitization and challenge. FK506 ointment (20 μl) was applied to the inner side of both ears 3 h before the challenge, and the ointment was removed with 70% ethanol just before challenge.

2.3. Effects on tuberculin-induced delayed allergic reaction in Lewis rats

Freund's complete adjuvant including 0.45 mg of killed *M. tuberculosis* (0.6 ml) divided into three parts was injected subcutaneously in Lewis rats for sensitization. Two weeks later, tuberculin-induced delayed allergic reaction was elicited by intradermal injection of PPD (50 μg) into the inner side of both ears in a volume of 50 μl. The delayed allergic reaction was assessed by measuring the increased ear thickness and the amount of dye leaked in the ear. In detail, at 24 h after the challenge, increase of ear thickness was measured with a dial thickness gauge, and then, rats injected intravenously with 0.5% Evans blue 4 h previously in a volume of 1 ml were killed and the amount of dye leaked in both ears was determined using a spectrometer. Rats were anesthetized with ether during the challenge.

Twenty microliters of FK506 ointment or alclometasone dipropionate ointment was applied to both sides of the ears 3 h before and 2 h after challenge. The ointment was removed with 70% ethanol just before challenge.

2.4. Effects on Egg albumin-induced passive cutaneous anaphylaxis reaction in Brown–Norway rats

Brown–Norway rats were sensitized by intradermal injection of 50 μl of eight-fold diluted antiserum of Sprague–Dawley rats containing anti-Egg albumin IgE, and 48 h later, the rats were challenged intravenously with 1 ml of saline containing 5 mg of Egg albumin and 5 mg of Evans blue for elicitation of passive cutaneous anaphylaxis. At 1 h after the challenge, the rats were killed and the amount of dye leaked in both ears was determined using a spectrometer. A total of 20 μl of FK506 ointment or alclometasone dipropionate ointment was applied to both sides of the ears 6 h before the challenge. Mice were anesthetized with ether during the sensitization.

2.5. Effect on tuberculin-induced delayed allergic reaction in locally passively sensitized BALB/c mice

BALB/c mice were sensitized in the same manner as mentioned in Section 2.2. After 16 days, spleen cells from the sensitized mice (donor mice) were harvested by the

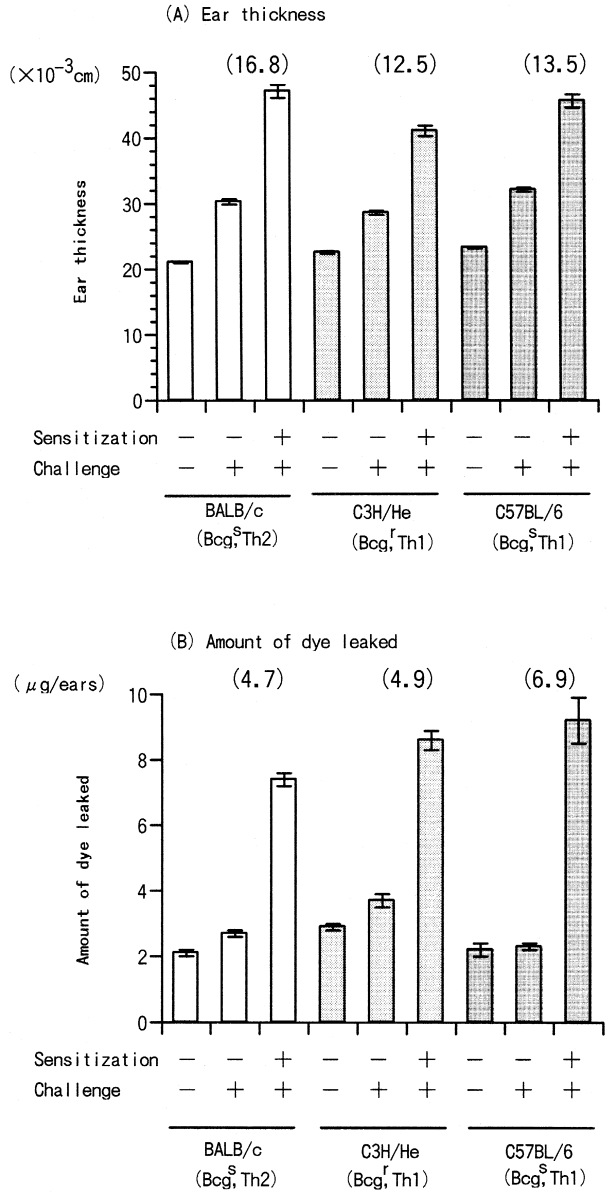

Fig. 1. Tuberculin-induced delayed allergic reaction in actively sensitized mice. Tuberculin-induced delayed allergic reaction was compared in BALB/c, C3H/He and C57BL/6 strain mice. The evaluation was performed by measuring (A) ear thickness and (B) amount of dye leaked in the ear. Each column represents the mean ± S.E.M. for 7–10 mice. Figures in parentheses represent the intensity of delayed allergic reaction calculated by subtracting mean of the group (Sensitization −, Challenge +) from mean of the group (Sensitization +, Challenge +).

conventional method, and a mixture of the cells (10^6 cells as lymphocytes) and PPD (5 μg) in a volume of 10 μl was quickly injected into the inner side of both ears of the same strain of naive mice (recipient mice) to elicit the tuberculin-induced delayed allergic reaction. The reaction at 24 h after the challenge was assessed by measuring the ear thickness and the amount of dye leaked in the ears in the same manner as mentioned in Section 2.2. To compare the effect of FK506 ointment on this reaction with that of FK506 ointment as studied in Section 2.2, application of the drug was done in the same manner as described in Section 2.2. The mice were anesthetized with ether during the sensitization and challenge.

2.6. Statistical analysis

The data were expressed as means ± S.E.M. Statistical significance of differences was assessed by Dunnett's or Tukey–Kramer's multiple comparison test following One-

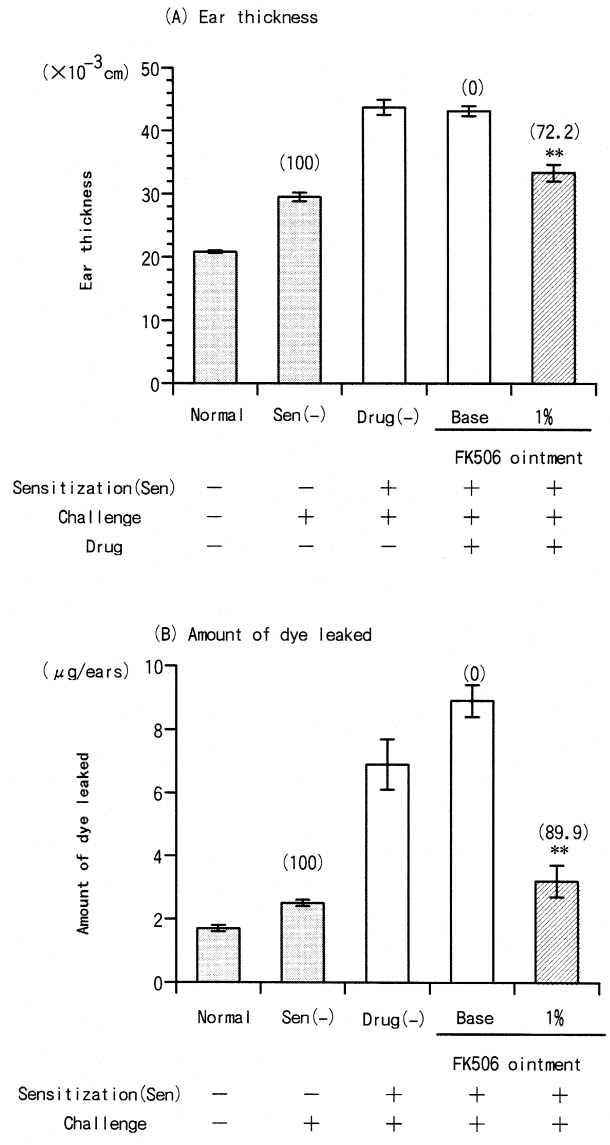

Fig. 2. Effects of FK506 ointment on tuberculin-induced delayed allergic reaction in actively sensitized BALB/c mice. FK506 ointment (20 μl) was applied to the inner side of both ears 3 h before the challenge. The ointment was removed with 70% ethanol just before the challenge. The evaluation was performed by measuring (A) ear thickness and (B) amount of dye leaked in the ear. Each column represents the mean ± S.E.M. for five to six mice. $^{**}P < 0.01$: significantly different from the FK506 ointment base group (Tukey–Kramer's multiple comparison test following One-way analysis of variance). Figures in parentheses represent percentage inhibition of delayed allergic reaction.

way analysis of variance. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Tuberculin-induced delayed allergic reaction in actively sensitized BALB/c, C3H/He and C57BL/6 mice

The tuberculin-induced delayed allergic reaction was compared in BALB/c, C3H/He and C57BL/6 strain mice. The delayed allergic reaction was elicited in all strains, and in terms of intensity, BALB/c mice and C57BL/6 mice had the strongest reaction, regarding ear thickness and amount of dye leaked, respectively. In all strains, ear thickness was a more sensitive measurement than the amount of dye, because the mice challenged but not sensitized showed an apparent increase only in ear thickness (Fig. 1).

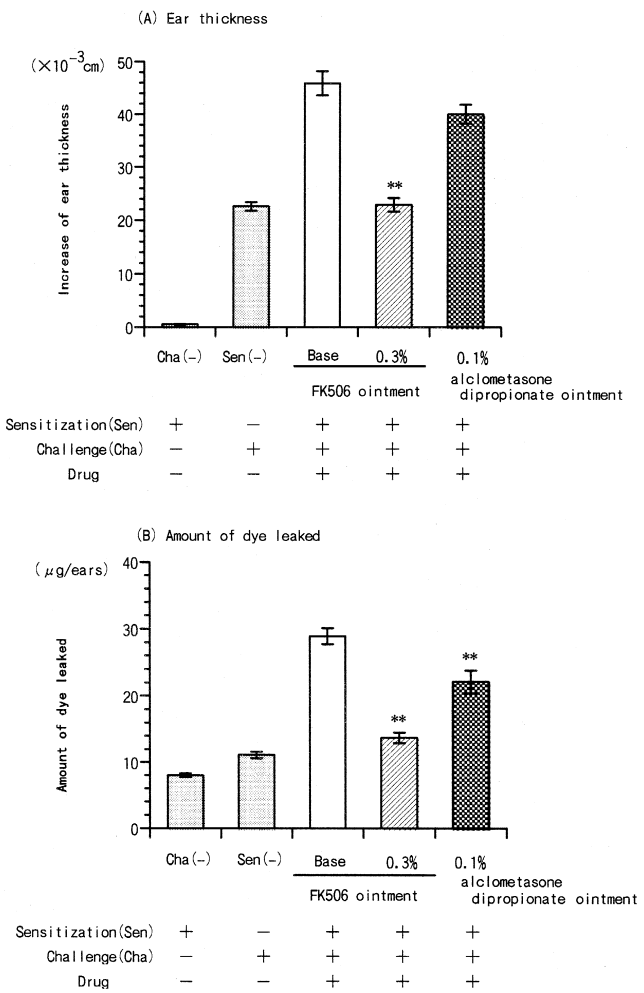

Fig. 3. Effects of FK506 ointment and alclometasone dipropionate ointment on tuberculin-induced delayed allergic reaction in Lewis rats. FK506 ointment or alclometasone dipropionate ointment (20 μl) was applied twice to both sides of the ears 3 h before and 2 h after the challenge. The ointment was removed with 70% ethanol just before the challenge. The evaluation was performed by measuring (A) ear thickness and (B) amount of dye leaked in the ear. Each column represents the mean ± S.E.M. for seven to eight mice. $^{**}P < 0.01$: significantly different from the FK506 ointment base group (Tukey–Kramer's multiple comparison test following One-way analysis of variance).

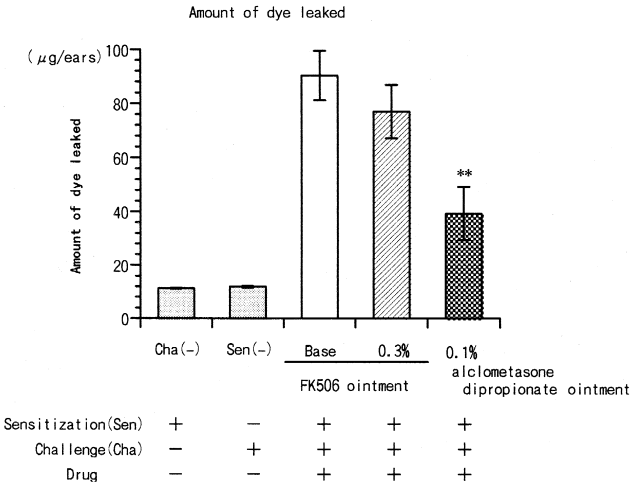

Fig. 4. Effects of FK506 ointment and alclometasone dipropionate ointment on Egg albumin-induced passive cutaneous anaphylaxis reaction in Brown–Norway rats. FK506 ointment or alclometasone dipropionate ointment (20 μl) was applied to both sides of the ears 6 h before the challenge. The evaluation was performed by measuring the amount of dye leaked in the ear. Each column represents the mean ± S.E.M. for five mice. $^{**}P < 0.01$: significantly different from the FK506 ointment base group (Dunnett's multiple comparison test following One-way analysis of variance).

3.2. Effect on tuberculin-induced delayed allergic reaction in actively sensitized BALB/c mice

FK506 ointment (1%) showed significant suppressive effects on the tuberculin-induced delayed allergic reaction in actively sensitized BALB/c mice. The percentage inhibition of FK506 ointment vs. ointment base was 72.2 and 89.9 for ear thickness and the amount of dye leaked, respectively (Fig. 2).

3.3. Effects on tuberculin-induced delayed allergic reaction in Lewis rats

The delayed allergic reaction to tuberculin was elicited in Lewis rats. FK506 ointment (0.3%) showed potent suppressive effects on both ear thickness and the amount of dye leaked. On the other hand, alclometasone dipropionate ointment (0.1%) partially inhibited the increase in amount of dye leaked, but not that of ear thickness. The inhibitory efficacy of FK506 ointment was apparently much greater than that of alclometasone dipropionate ointment (Fig. 3).

3.4. Effects on Egg albumin-induced passive cutaneous anaphylaxis reaction in Brown–Norway rats

The passive cutaneous anaphylaxis reaction to Egg albumin was elicited in Brown–Norway rats. Alclometasone dipropionate ointment (0.1%) significantly suppressed the increase in amount of dye leaked in this passive cutaneous anaphylaxis reaction, whereas FK506 ointment (0.3%) did not inhibit the reaction (Fig. 4).

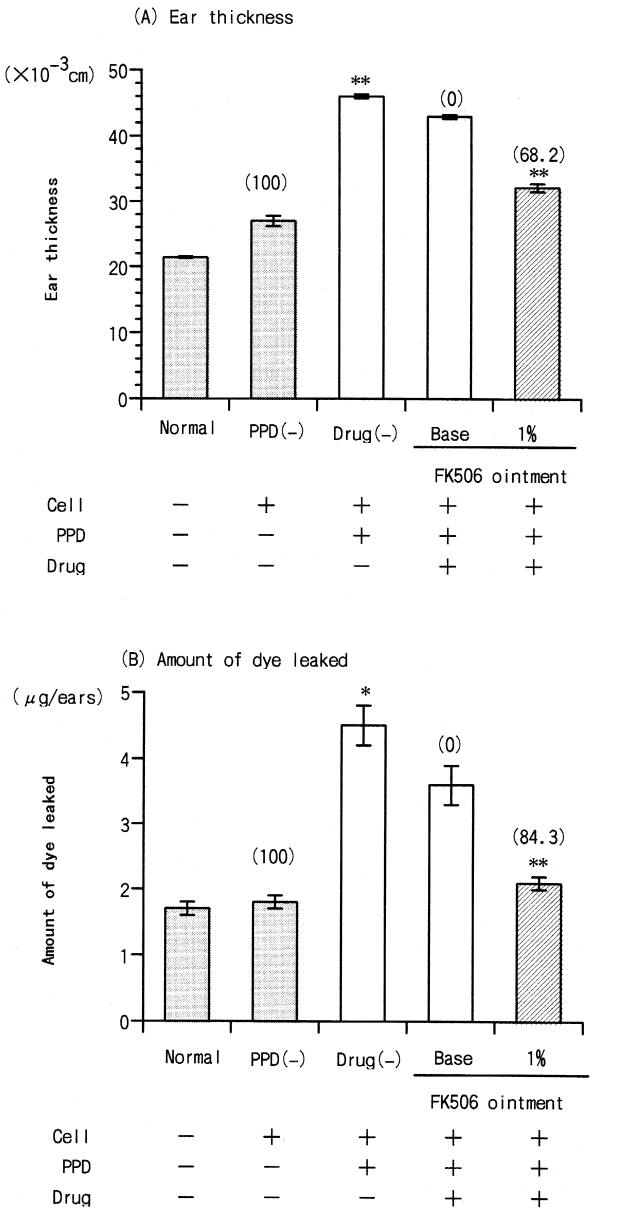

Fig. 5. Effects of FK506 ointment on tuberculin-induced delayed allergic reaction in locally passively sensitized BALB/c mice. Tuberculin-induced delayed allergic reaction was elicited by injecting a mixture of PPD and spleen cells harvested from mice sensitized with tuberculin (donor mice) into the ears of the same strain of naive mice (recipient mice). FK506 ointment (20 μl) was applied to the inner side of both ears 3 h before the challenge. The ointment was removed with 70% ethanol just before the challenge. The evaluation was performed by measuring (A) ear thickness and (B) amount of dye leaked in the ear. Each column represents the mean ± S.E.M. for four to five mice. $^{*}P < 0.05$, $^{**}P < 0.01$: significantly different from the FK506 ointment base group (Tukey–Kramer's multiple comparison test following One-way analysis of variance). Figures in parentheses represent percentage inhibition of delayed allergic reaction.

3.5. Effect on tuberculin-induced delayed allergic reaction in locally passively sensitized BALB/c mice

We studied the site of action of FK506 ointment on tuberculin-induced delayed allergic reaction in mice. FK506 ointment (1%) significantly suppressed the delayed allergic reaction in locally passively sensitized BALB/c mice. The percentage inhibition for FK506 ointment vs. ointment base was 68.2 and 84.3 for ear thickness and amount of dye leaked, respectively (Fig. 5).

4. Discussion

In this work, we focused on the delayed allergic reaction. First, the tuberculin-induced delayed allergic reaction was compared in three strains (BALB/c mice, C3H/He mice and C57BL/6 mice) to select the optimal mouse strain for evaluation of FK506 ointment. It has been reported that BALB/c mice, C57BL/6 mice and C3H/He mice are strains that are susceptible (Bcg^s), susceptible (Bcg^s) and resistant (Bcg^r) to *M. bovis* (BCG), respectively (Gros et al., 1981; Denis et al., 1986). On the other hand, C57BL/6 mice, C3H/He mice and BALB/c mice are strains that are susceptible to the Th1, Th1 and Th2 immune response, respectively (Martinez-Abrajan, 1993; Scott et al., 1996). Because the intensity of the ear thickness in C3H/He mice (Bcg^r, Th1) was similar to that in C57BL/6 mice (Bcg^s, Th1) and also the differences between BALB/c mice (Bcg^s, Th2) and C57BL/6 mice (Bcg^s, Th1) were not clear, it is thought that the elicitation of this tuberculin-induced delayed allergic reaction was not necessarily influenced by its susceptibility to the Th2 or Th1 response and the sensitivity to tuberculin. In this case, as optimal strain for tuberculin-induced delayed allergic reaction, we selected BALB/c mice which showed the strongest delayed allergic reaction for ear thickness since this was a more sensitive parameter than the amount of dye.

FK506 ointment showed a significant suppressive effect on the tuberculin-induced delayed allergic reaction in actively sensitized BALB/c mice, as in a previous experiment by Sengoku et al., 1998. We used a concentration of PPD 20 times higher than that of the previous experiment. The result demonstrated that FK506 ointment can also inhibit a more severe tuberculin-induced delayed allergic reaction in mice.

Then, to further clarify the inhibitory action of FK506 ointment, the delayed allergic reaction was performed in

Lewis strain rats which are especially susceptible to induction of the Th1 immune response (Groen et al., 1993). It was reported that Lewis rats express a more extensive allergic contact response to dinitrofluorobenzene than do rats of the Brown–Norway strain (Peszkowski et al., 1994). On the other hand, Brown–Norway strain rats, which are susceptible to induction of Th2 immune response (Groen et al., 1993; Kiely et al., 1995), have the propensity to produce high levels of IgE in response to antigen (Pauwels et al., 1979) and are highly sensitive to the action of substances on mast cell degranulation (Oliveira et al., 1995; Hodson and Oliveira, 1996). These rats were used in the experiment with the passive cutaneous anaphylaxis reaction, a typical model of immediate allergic reaction. FK506 ointment showed significant suppressive effects on the delayed allergic reaction and its inhibitory action was much stronger than that of alclometasone dipropionate ointment, a so-called medium class steroid ointment, whereas we previously reported that the inhibitory actions of FK506 ointment in mice were equal to that of the steroid (Sengoku et al., 1998). Accordingly, the result for rats agrees with those of the FK506 ointment clinical study, and therefore, the tuberculin-induced delayed allergic reaction model in rats would be a useful prospect for assessing the clinical effect of a drug.

On the other hand, FK506 ointment did not inhibit the immediate allergic reaction in rats, similar to its effect in mice as reported previously (Sengoku et al., 1998). This finding was in contrast to the steroid effect. These results led to the suggestion that the main inhibitory mechanism of FK506 ointment for atopic dermatitis involved the Th1 allergic reaction underlying the chronic phase of atopic dermatitis.

Next, we further investigated the site of action of FK506 ointment on the delayed allergic reaction. The efficacy of FK506 ointment in locally passively sensitized mice was almost similar to that in actively sensitized mice. Accordingly, it is speculated that FK506 ointment inhibits the activation of the Th1 cells accumulated in the dermis immediately after challenge, and that the inhibitory mechanism may be involved in the in vitro findings that FK506 inhibited T cell-derived production of cytokines such as interleukin-2 and interferon-γ (Andersson et al., 1992; Schreiber and Crabtree, 1992; Rao, 1994). Of course, FK506 may target Langerhans cells, which are an antigen-presenting cell, or somewhere downstream of the Th1 cells activation, except cytokines production.

The inhibitory mechanisms of topical FK506 on the induction phase of delayed allergic reactions have recently been reported upon (Homey et al., 1998; Salerno et al., 1998). We now described for the first time the possible inhibitory mechanism of FK506 ointment action on the effector phase of delayed allergic reaction.

In conclusion, FK506 ointment is speculated to suppress the chronic phase of atopic dermatitis mainly by inhibiting the activation of Th1 cells already accumulated in the dermis. Therefore, FK506 ointment is expected to exert a therapeutic effect locally against the chronically incurable atopic dermatitis.

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