Effect of Tacrolimus Hydrate (FK506) Ointment on Spontaneous Dermatitis in NC/Nga Mice

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ABSTRACT—The effect of tacrolimus hydrate (FK506) ointment on spontaneous dermatitis in NC/Nga (NC) mice was examined. FK506 ointment (0.1–1%) suppressed the development of dermatitis and was also therapeutically effective against established dermatitis. Increases in CD4-positive T cells (helper T cells), mast cells, eosinophils and immunostaining of interleukin (IL)-4, IL-5 and IgE were confirmed in the skin of the NC mice, and FK506 ointment suppressed all of these changes. Increased plasma IgE was also confirmed in the NC mice, and treatment with FK506 ointment reduced the plasma IgE level. These results suggested that FK506 suppressed the dermatitis by inhibiting the activation of inflammatory cells and by blocking the cytokine network in the skin of the NC mice. The commercially available steroid ointments showed only marginal effect on the development of dermatitis and showed some signs of side effects such as alopecia or atrophy of the skin. The effect of the steroids might have been masked by these side effects because the steroids showed similar inhibitory effects on the skin histopathological changes and the increase of plasma IgE. From these results, FK506 ointment can be expected to be a useful drug for atopic dermatitis.

Keywords: FK506, Atopic dermatitis, T cell, Mast cell, Interleukin-4

Spontaneous severe dermatitis has already been reported in NC/Nga (NC) mice (1, 2). The mechanisms of the development of this dermatitis has not been clarified, but immunological factors might contribute to its progress (2, 3). We also have observed that dermatitis appeared about 8 weeks after birth when mice were raised in conventional rearing conditions and that plasma IgE increased to high levels along with the development of dermatitis. Histopathological studies also showed that inflammatory cells such as CD4-positive T cells, mast cells and eosinophils were increased in the skin of NC mice (4). Furthermore, it is suggested that both dermatitis and the level of plasma IgE of NC mice are regulated genetically (5). All of these features are very similar to those of patients with atopic dermatitis (6, 7). It is suggested that there is an immunological disorder in NC mice and that such mice may be suitable models of atopic dermatitis in humans.

FK506, a new macroline immunosuppressive agent (8, 9), is currently used as an immunosuppressant for liver and kidney transplantation worldwide. FK506 inhibits cytokine production of T cells (10, 11) and histamine release from mast cells (12, 13), as well as delayed type allergic reaction in animal models (14) and humans (15). Since these results suggested that FK506 had an inhibitory activity on atopic dermatitis, FK506 ointment has been developed, and found to be effective on human atopic dermatitis (16). In this paper, we describe the effects of FK506 ointment on dermatitis in NC mice and discuss the mechanisms of the action of FK506 and the mechanisms of the appearance of dermatitis in these mice.

MATERIALS AND METHODS

Animals

Male and female NC mice were bred in the Department of Laboratory Animal Science, College of Agriculture, University of Osaka Prefecture.
Drugs
FK506 ointment was prepared at Fujisawa Pharmaceutical Co., Ltd. Corticosteroid ointments betamethasone valerate ointment (Rinderon®-V ointment, 0.12%) and alclometasone dipropionate ointment (Almeta® ointment, 0.1%) were purchased from Shionogi Pharmaceutical Co., Ltd. (Osaka).

Evaluation of inhibitory effect on dermatitis
Effect on developing dermatitis: Five- to eight-week-old NC mice with no skin symptoms were used. One hundred milligrams of ointment was applied to the skin of the head and neck two times a week, on Monday or Tuesday and Thursday or Friday. The severity of dermatitis was assessed once a week by the following scoring procedure: No symptoms, 0; mild inflammation or wound, 1; moderate inflammation or wound or mild hemorrhage, 2; severe inflammation or hemorrhage or ulcer or loss of ears, 3. After about 9 weeks of treatment, the animals were anesthetized with ether and blood was taken by cardiac puncture with heparinized syringes. The number of red blood cells was assessed using a hemocytometer (Sysmex E-4000; Toa Medical Electronics Co., Ltd., Kobe). Plasma IgE, IgG1, and IgG2a levels were assessed by enzyme immuno-assay according to the previously described method (17) using monoclonal antibody (Yamasa Shoyu Co., Ltd., Choshi).

Effect on established dermatitis: Eleven- to fifteen-week-old NC mice with dermatitis were used. Ointment, scoring criteria and treatment schedule were the same as above.

Histopathological study
Whole heads of the animals were fixed in 10%-buffered formalin solution and decalcified in 10%-formic acid-formalin solution. A block of the forehead skin was removed and embedded in paraffin by the conventional method, cut in 3- and 6-μm sections, and stained with hematoxylin-eosin (HE) and toluidine blue, respectively.
A piece of fresh skin from between the ears of each animal was embedded in OCT compound (Miles, Inc., Elkhard, IN, USA), snap frozen in dry ice-acetone, and then stored at -80°C until use. Frozen sections, cut in 5-μm slices, were fixed in acetone for 10 min. After pretreatment with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase, the preparations were washed with phosphate-buffered saline (PBS) twice and then treated with blocking medium (10% normal goat serum in PBS). Rat monoclonal antibodies against mouse CD4, interleukin (IL)-4, IL-5 and IgE, purchased from Pharmingen (San Diego, CA, USA), were applied for 1 or 3 hr in PBS with 1% bovine serum albumin; and after washing, goat anti-rat IgG conjugated with peroxidase (Jackson, West Grove, PA, USA) was overlaid for 30 min. Visualization of the reaction products was performed with 3-amino-9-ethylcarbazole. For staining of eosinophils, frozen sections were treated by O-phenylene diamine (OPD) after fixation using 10%-buffered formalin. Mast cells stained by toluidine blue were counted by cell number and CD4-positive cells, eosinophils, IL-4, IL-5 and IgE were graded by the following criteria: no staining, 0; slight staining, 1; moderate staining, 2; marked staining, 3; very strong staining, 4.

Statistical analyses
Data was expressed as the mean±S.E.M. Statistical significances of differences were assessed by Dunnett’s multiple comparison test following Kruskal-Wallis test or one way analysis of variance and Student’s or Aspin-Welch’s t-test for two sample comparison. P values less than 0.05 were considered statistically significant.

RESULTS
Effects of FK506 ointment and steroid ointments on the development of dermatitis
NC mice, 6- to 8- (FK506 study) or 5- to 8- (steroids study) week-old, with no superficial dermatitis were used. In the untreated mice, dermatitis appeared and increased gradually from the commencement of the study and reached peak levels of inflammation score in weeks 4 to 6 (Figs. 1 and 2). In the ointment base-treated control group, slight inhibition of dermatitis was observed, and

Fig. 1. Effect of FK506 ointment on the development of dermatitis in NC mice. Ointment (100 mg) was applied to the head and neck of 6- to 8-week-old mice for about 9 weeks. ○: no treatment (n=11), □: ointment base (n=12), ■: 0.1% FK506 ointment (n=11), ▲: 0.3% FK506 ointment (n=11), ▼: 0.5% FK506 ointment (n=11), ●: 1% FK506 ointment (n=11). Values are means±S.E.M.. *: Significantly different from the ointment base-treated group at P<0.05 (Dunnett’s multiple comparison test following Kruskal-Wallis test).
in all the FK506 ointment (0.1–1%)-treated groups, only slight skin symptoms were observed throughout the observation period (Fig. 1). The two commercially available steroid ointments, betamethasone valerate and alclometasone dipropionate, did not show any clear inhibitory effects (Fig. 2) and even showed some incidence of side effects such as alopecia and atrophy of the skin.

**Effect of FK506 ointment on established dermatitis**

Mild to severe inflammation of the skin developed in the 11- to 15-week-old NC mice, and the mean score of the symptom in each group was more than 1.5 before commencement of the study. In the FK506 ointment-treated mice, the score decreased gradually during 3 weeks, and significant inhibition was observed in the 0.5% FK506 ointment-treated group compared with the ointment base-treated group (Fig. 3).

**Effects of FK506 and steroid ointments on plasma immunoglobulin level and red blood cells**

Plasma IgE, IgG1, and IgG2a levels were increased in the 15- to 17-week-old untreated group compared with younger 6- to 8-week-old animals (Table 1). FK506 ointment showed a concentration-dependent decrease of plasma IgE and also plasma IgG1, but without concentration

<p>| Table 1. Effects of FK506 ointment on plasma IgE, IgG1, and IgG2a levels in NC mice |
|---------------------------------|-----------------|---------------|--------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>IgE (µg/ml)</th>
<th>IgG1 (µg/ml)</th>
<th>IgG2a (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ointment base (15–17)†</td>
<td>12</td>
<td>70.48±14.36</td>
<td>1698±300</td>
<td>2267±427</td>
</tr>
<tr>
<td>FK506 ointment (15–17)</td>
<td>11</td>
<td>35.85±14.52</td>
<td>776±156*</td>
<td>1700±121</td>
</tr>
<tr>
<td>0.3% (15–17)</td>
<td>11</td>
<td>19.46±7.91**</td>
<td>721±177**</td>
<td>1355±107</td>
</tr>
<tr>
<td>0.5% (15–17)</td>
<td>11</td>
<td>8.19±2.91**</td>
<td>837±137*</td>
<td>1660±72</td>
</tr>
<tr>
<td>1% (15–17)</td>
<td>11</td>
<td>7.72±1.68**</td>
<td>981±222</td>
<td>1339±206</td>
</tr>
<tr>
<td>No treatment (15–17)</td>
<td>11</td>
<td>104.39±14.88</td>
<td>1698±231</td>
<td>1921±421</td>
</tr>
<tr>
<td>(6–8)</td>
<td>10</td>
<td>7.14±3.05</td>
<td>309±70*</td>
<td>425±56*</td>
</tr>
</tbody>
</table>

†Figures in parentheses are the age in weeks of mice when blood was taken. Ointment (100 mg) was applied to the head and neck of 6- to 8-week-old NC mice for about 9 weeks. Values are means±S.E.M. * **: Significantly different from the ointment base-treated group at P<0.05 and P<0.01, respectively (Dunnett's multiple comparison test following one way analysis of variance).

‡: Significantly different from 15- to 17-week-old mice at P<0.01 (Student's t-test or Aspin Welch's t-test).
dependency in the latter (Table 1). Both of the steroid ointments also had a decreasing effect on plasma IgE, and alclometasone dipropionate ointment showed a slight inhibitory effect on IgG1, but betamethasone valerate ointment did not (Table 2). On the other hand, plasma IgG2a was not decreased by FK506 and either steroid ointment (Tables 1 and 2). In the 15- to 17-week-old untreated NC mice a decrease of red blood cells was observed, but FK506 did not show any effect on the cell number (data not shown).

**Histopathological study**

Even in the younger animals (6- to 8-week-old) that superficially appeared to have no dermatitis, slight infiltration of the cells into the dermis was observed in the section stained by HE. In the untreated animals, 15- to 17-week-old, skin ulcer, thickening of the epidermis and infiltration of many kinds of cells into the dermis was observed (data not shown). In the sections stained by toluidine blue or OPD and immunostaining, mast cells, eosinophils, CD4-positive T cells, IgE, IL-4 and IL-5

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**Table 2.** Effects of betamethasone valerate ointment and alclometasone dipropionate ointment on plasma IgE, IgG1, and IgG2a levels in NC mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>IgE (µg/ml)</th>
<th>IgG1 (µg/ml)</th>
<th>IgG2a (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (14–17)</td>
<td>16</td>
<td>81.65 ± 7.96</td>
<td>2955 ± 286</td>
<td>2367 ± 214</td>
</tr>
<tr>
<td>Betamethasone valerate ointment 0.12% (14–17)</td>
<td>15</td>
<td>32.03 ± 3.39**</td>
<td>2386 ± 247</td>
<td>2307 ± 318</td>
</tr>
<tr>
<td>Alclometasone dipropionate ointment 0.1% (14–17)</td>
<td>17</td>
<td>49.30 ± 5.12**</td>
<td>2136 ± 235*</td>
<td>2277 ± 155</td>
</tr>
</tbody>
</table>

*Figures in parentheses are the age in weeks of mice when blood was taken. Ointment (100 mg) was applied to the head and neck of 5- to 8-week-old NC mice for about 9 weeks. Values are means ± S.E.M. **: Significantly different from the ointment base-treated group at P < 0.05 and P < 0.01, respectively (Dunnnett’s multiple comparison test following one way analysis of variance).
(b) Mast cells

- no treatment (6- to 8-week-old)
- no treatment (15- to 17-week-old)
- ointment base (15- to 17-week-old)
- FK506 (1%) ointment (15- to 17-week-old)

(c) IL-4

- no treatment (6- to 8-week-old)
- no treatment (15- to 17-week-old)
- ointment base (15- to 17-week-old)
- FK506 (1%) ointment (15- to 17-week-old)

Fig. 4.
Fig. 5. Effects of FK506 and steroid ointments on CD4-positive T cells (a), mast cells (b) and eosinophils (c) in dermis of NC mice. Values are means±S.E.M. *: Significantly different from the ointment base-treated group at P<0.05 and P<0.01, respectively. **: Significantly different from the untreated group at P<0.01 (Dunnett's multiple comparison test following Kruskal-Wallis test or one way analysis of variance).
Fig. 6. Effects of FK506 and steroid ointments on IgE (a), IL-4 (b) and/or IL-5 (c) staining in dermis of NC mice. Values are means ± S.E.M. * * *: Significantly different from ointment base-treated group at $P<0.05$ and $P<0.01$, respectively. # #: Significantly different from untreated group at $P<0.05$ and $P<0.01$, respectively (Dunnnett's multiple comparison test following Kruskal-Wallis test).
were increased in the dermis. Typical results of staining
of CD4-positive T cells, mast cells and IL-4 are shown in
Fig. 4. Treatment with FK506 ointment for about 9 weeks
showed an inhibitory effect on all of these changes, but
dose-responsiveness was not always observed (Figs. 5
and 6). The steroid ointments showed similar inhibitory
effects on most of these features, except IL-5 for which
there was no appropriate antibody (Figs. 5 and 6). Since
the intensity of immunostaining of CD8-positive cells,
IL-2 and interferon (IFN)-γ was very weak even in the
dermis of the untreated animals, the effects of the oint-
ments were not studied (data not shown).

DISCUSSION

As we have already reported (4), severe dermatitis
developed in NC mice about 8 weeks after birth when
the animals were raised in conventional rearing condi-
tions. FK506 ointment showed inhibitory activity on the
development of dermatitis and also showed therapeutic
effect on established dermatitis in these mice.

As we also reported, pathophysiological assessment of
the skin of NC mice showed that the numbers of CD4-
positive T cells, mast cells and eosinophils were increased
in the dermis of the NC mice (4). We confirmed these
changes in this study, as well as an increase in the inten-
sity of immunological staining of IL-4, IL-5 and IgE; and
these changes were quantitatively assessed by scoring the
grade of the staining. Since these pathophysiological
changes are less prominent in younger animals which
have no inflammation in the skin, dermatitis and histopathological changes are thought to be correlated
with each other.

Atopic dermatitis is reported to be induced by the acti-
vation of inflammatory cells such as T cells, mast cells
and eosinophils, and the cytokine network of these cells is
suggested to be very important (6, 7). From the results of
this study, pathophysiological changes of the dermis in
NC mice are thought to be very similar to those in human
atopic dermatitis patients. FK506 ointment showed the
suppressing effects on all of these changes in NC mice, but
it was difficult to determine the main target cells of FK506
action from these results. FK506 has been reported to
have inhibitory effects on the activation of inflammatory
cells, such as T cells (10, 11) and mast cells (12, 13) in
animals and humans in vitro. Thus we consider that the
clinical effect of FK506 ointment on atopic dermatitis is
achieved by inhibition of the activation of these cells.

As reported by Tamada (18), red blood cells were
decreased in NC mice. Since FK506 showed no effect on
such anaemia in our study, it seemed that FK506 was not
effective on some of the pathological changes in these
mice.

The two corticosteroid ointments showed only a mar-
ginal effect on the development of dermatitis. We do not
know the reason but side effects such as alopecia and
atrophy of the skin may have masked the anti-dermatitis
activity, since the steroid ointments showed an inhibi-
tory effect on the changes observed by histopathological
studies.

Plasma IgE level was elevated even in the younger mice,
and the increment coincided with the development of
dermatitis in older mice. Although the level of plasma
IgE clearly declined in the FK506- and steroids-treated
animals, the mechanisms by which this was achieved was
not clarified. The activity of inflammatory cells such as
mast cells and Langerhans cells may be suppressed by
lowering plasma IgE level. In fact immunostaining of
IgE in the dermis was decreased in both the FK506- and
steroids-treated NC mice, even though this may be related
with the decrease of mast cell numbers. Plasma IgG1 level
was also decreased, but IgG2a level was not affected. Since
it was reported that IgE and IgG1 are regulated by IL-4
from T helper type 2 (Th2) cells (19) and IgG2a by IFN-γ
from Th1 cells (20) in mice, FK506 might suppress the
Th2 cells selectively. Since the intensity of immunostain-
ing of IL-2 and IFN-γ was weak, Th2 cells might be es-
specially augmented in NC mice. On the other hand, mast
cells are reported to produce other cytokines including
IL-4 (21, 22). It was also reported that mast cells in the
skin of NC mice produced IL-4 (4). FK506 might suppress
the production of IL-4 from the mast cells. This should
be made clear by in vitro studies.

FK506 has been shown to have less non-specific cyto-
toxic effect on stem cells than that of the steroids (9), and
skin atrophy by the steroid ointments has been reported
in animals and humans (23, 24). We also observed the
changes in the steroid-treated rats, but not in the FK506-
treated rats (25). These results suggest that the action of
FK506 is specific to the inflammatory cells, and therefore,
the clinical side effects of FK506 will be less than those of
steroids in atopic dermatitis patients.

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