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Spontaneous scratching behavior in MRL/*lpr* mice, a possible model for pruritus in autoimmune diseases, and antipruritic activity of a novel κ-opioid receptor agonist nalfurafine hydrochloride

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Abstract

Pruritus is a common, distressing and difficult to manage complication of many autoimmune diseases. A suitable animal model of autoimmune disease associated pruritus would contribute to a better understanding of the pathophysiology of this symptom and lead to the development of safe and effective antipruritic agents. We noticed spontaneous scratching behavior in aged MRL/*lpr* mice, a model of autoimmune disease. This scratching behavior was observed in a specific pathogen-free environment and was more frequent in female mice. In contrast to animal models of dermatitis; NC/Nga mice, the serum IgE and IgG_1 levels in MRL/*lpr* mice were not elevated. These features indicate that this scratching behavior is similar to human autoimmune disease associated pruritus. The antipruritic effects of an antihistamine (chlorpheniramine), an opioid receptor antagonist (naltrexone), and a novel κ-opioid receptor agonist (nalfurafine hydrochloride [TRK-820]) were evaluated. The frequency of scratching was not reduced by oral administration of chlorpheniramine, suggesting that the behavior is antihistamine-resistant. The oral administration of nalfurafine and subcutaneously administered naltrexone inhibited the scratching behavior without causing gross behavioral changes. In conclusion, MRL/*lpr* mice scratching behavior is a suitable model of pruritus that occurs in autoimmune diseases, and nalfurafine was shown to be efficacious against this behavior suggesting that it may be beneficial in patients with autoimmune disease associated pruritus.

Keywords: Pruritus; MRL/*lpr* mouse; Scratching behavior; κ-opioid agonist

1. Introduction

Pruritus is a common symptom in autoimmune diseases such as systemic lupus erythematosus (Kapadia and Haroon, 1996), pemphigus vulgaris (Woldegiorgis and Swerlick, 2001), Sjögren–Larsson syndrome (Willemsen et al., 2001), multiple sclerosis (Osterman, 1976), and primary biliary cirrhosis (Heathcote, 2000; Talwalkar et al., 2003). The pathogenesis of this symptom is unknown and there are limited treatment options available, e.g. antihistamines are not effective (Levy and Lindor, 2003); anion exchange resins cholestyramine (Datta and Sherlock, 1963) and hepatic enzyme-inducing agents rifampin are beneficial in only a small portion of patients (Ghent and Carruthers, 1988; Bachs et al., 1992) against pruritus in primary biliary

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cirrhosis. Unfortunately, for many patients these agents are ineffective and better methods of treating this symptom need to be developed.

Animal models have recently been used to develop novel therapies for pruritus. For example, scratching behavior in mice after the administration of pruritogens, substance P or histamine, has been used to test the efficacy of antipruritic agents (Andoh et al., 1998; Kuraishi et al., 1995). NC/Nga mice have been used as an animal model of atopic dermatitis because when kept in a non-sterile environment these mice develop a helper T cell type 2 (Th2) immune response, immunoglobulin (Ig) E hypersynthesis, inflammatory skin lesions and scratching behavior (Maekawa et al., 2002; Takano et al., 2003). Although NC/Nga mice have been used as a model of atopic dermatitis (Hiroi et al., 1998), they are unlikely to be useful for autoimmune diseases that have a predominantly Th1 immune response. In order to elucidate the mechanism of pruritus in autoimmune disease and develop more effective treatment, it is necessary to establish a suitable animal model.

MRL/Mp-*lpr/lpr* (MRL/*lpr*) mice are a unique strain with a deficit in Fas-mediated apoptosis and develop severe autoimmune diseases such as glomerulonephritis, polyarthritis, and arteritis (Hamano et al., 1993; Okamoto et al., 2004). We recently observed scratching behavior and abrasions in the ears and rostral back of female MRL/*lpr* mice at approximately 18 weeks of age. Furthermore, this scratching behavior in MRL/*lpr* mice occurred under a specific pathogen-free environment. The first purpose of our study was to clarify the features of spontaneous scratching behavior in MRL/*lpr* mice and to evaluate the possibility of using these mice as a model of pruritus associated with autoimmune disease.

It was recently reported that κ-opioid receptor agonists inhibit pruritogen-induced scratching behavior in mice (Kamei and Nagase, 2001). A novel κ-opioid receptor agonist, nalfurafine hydrochloride (TRK-820), *N*-[(5R,6R, 9R,13S,14S)-17-Cyclopropylmethyl-4,5-epoxy-3,14-dihydroxymorphinan-6-yl]-(E)-3-(3-furyl)-*N*-methyl-2-propenamide monohydrochloride, was reported to decrease antihistamine-sensitive and -resistant pruritus in mice (Togashi et al., 2002; Umeuchi et al., 2003), and has clinical efficacy against uremic pruritus in humans (Sorbera et al., 2003). Hence, our second purpose of the present study is to clarify the effect of nalfurafine on spontaneous scratching behavior in MRL/*lpr* mice, compared with antihistamine and opioid antagonist.

2. Materials and methods

2.1. Animals

Male and female MRL/*lpr* mice (Charles River Japan, INC., Yokohama, Japan) and male ICR mice (Japan SLC, Inc., Shizuoka, Japan) were kept in a specific pathogen-free environment, male NC/Nga (Japan SLC, Inc.) mice in a non-sterile environment. MRL/*lpr* and NC/Nga mice were housed alone; ICR mice were housed 5 per cage. All the mice were under a controlled 12-h light–dark cycle and allowed free access to food pellets and tap water. This study was conducted in accordance with the guidelines for the care and use of laboratory animals in Toray Industries, Inc.

2.1.1. Evaluation of scratching behavior

The scratching behavior was observed according to the method described (Kuraishi et al., 1995). Briefly, mice were individually placed in sections of an observation cage (each cage consisted of four sections $10 \times 14 \times 30$ cm in size) and allowed to acclimate to their environment. Their behavior was recorded for 60 min using a video camera with no persons present in the room. All behavioral experiments were conducted in a specific pathogen-free environment and carried out between 9 am and 5 pm. Each recording was played back to count the number of scratches. Mice generally scratch with their hind paws several times a second, and therefore a series of these scratches was counted as one scratch event. The spontaneous scratching behavior was evaluated in male and female MRL/*lpr* mice every 2 weeks at 8 to 20 weeks of age.

2.1.2. Observation of dermal scratch marks

The dermal abrasions on the ear and rostral back of male and female MRL/*lpr* mice ($n=6$) were observed every 2 weeks in mice 8 to 20 weeks of age. The abrasion score was calculated as the total of a two-part score (ear plus rostral back) as follows: ear — 0, fine; 1, abrasion; 2, slight bleeding and abrasion; 3, scab and abrasion; 4, serious organization incrustation; rostral back — 1, good fur and normal skin; 2, slight alopecia and abrasion; 3, scab and abrasion. The highest possible combined score was 7.

2.1.3. Measurement of immunoglobulin levels

Serum levels of IgE and IgG were measured every 2 weeks in male and female MRL/*lpr* mice ($n=6$) 8 to 20 weeks of age. MRL/*lpr* mice were anesthetized slightly with ether and samples of blood (100 μl) were collected from the orbital venous plexus using a capillary glass tube. Natural coagulation was carried out at room temperature for about 1 h and the samples were centrifuged at 4 °C at 500 *g* for 15 min. The serum was stored at −80 °C until use. Total IgE and total IgG were measured according to standard methods using enzyme immunoassay kits (Yamasa-shoyu, Tokyo, Japan, and Bethyl Laboratory Inc., Montgomery, TX, respectively). Immunoglobulin subtypes such as G_1 and G_{2a} were also measured in MRL/*lpr* mice and the results were compared with those from NC/Nga and ICR mice at 20 weeks of age. Blood from NC/Nga and ICR mice were taken from the abdominal aorta and the serum was obtained using the method described above.

2.1.4. Evaluation of antipruritic activity of test compounds

The antipruritic activity of nalfurafine, chlorpheniramine and naltrexone was tested on female MRL/*lpr* mice at 18–20 weeks of age. Nalfurafine hydrochloride (synthesized by Toray Industries Inc., Tokyo, Japan) and (+)-chlorpheniramine maleate salt (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water; naltrexone hydrochloride dehydrate (Sigma-Aldrich) was dissolved in saline. The evaluation was carried out using a dose-up test using the same mice for a given compound. Day 1: the vehicle was

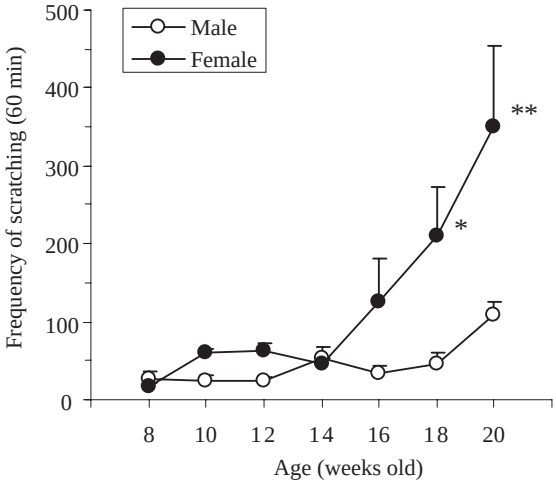

Fig. 1. Spontaneous scratching behavior in MRL/*lpr* mice. The scratching behavior of non-treated MRL/*lpr* mice (male; $n=6$, and female; $n=6$) was measured every 2 weeks from 8 to 20 weeks of age in a specific pathogen-free environment. The ordinate represents the number of scratches measured during a 60 min (means and S.E.M.) videotape in which their behavior was recorded. Males are shown in open circles and females in closed circles. $^{*}P<0.05$, $^{**}P<0.01$ when compared with the frequency of scratching at 8 weeks of age by multiple comparisons of time effects during longitudinal measurement.

administered and the frequency of spontaneous scratching behavior for 60 min was measured as baseline. From day 2 to day 5: the test compound, oral chlorpheniramine (3, 10, 30 mg/kg, $n=8$), oral nalfurafine (3, 10, 30 and 100 μg/kg, $n=8$) or subcutaneous naltrexone (3 mg/kg, $n=8$) was administered. Chlorpheniramine, nalfurafine and naltrexone were administered 60, 30 and 30 min before the recording, respectively. The scratching behavior was quantified as described above.

2.2. Statistical analysis

Statistical analysis was performed using SAS System Version 8.2 (SAS Institute Japan Inc., Tokyo, Japan). Statistical analysis was carried out by multiple comparisons of time effects determined by longitudinal measurement versus the parameter of interest at 8 weeks of age. Unpaired *t*-tests or Welch tests were used for statistical analysis of the immunoglobulin levels in murine strains. The efficacy of test compounds was examined by performing multiple comparisons of time effects for longitudinal measurements or by using the paired *t*-test where appropriate.

3. Results

3.1. Increased pruritus-associated response with aging in MRL/lpr mice

Scratching behavior was assessed in 8- to 20-week-old male and female MRL/*lpr* mice ($n=6$) every 2 weeks (Fig. 1). The frequency of scratching for 60 min in females gradually increased at 10 weeks of age and significantly increased at 18 and 20 weeks of age (60.5 ± 5.2, 209.5 ± 62.5 and 349.5 ± 104.9, respectively). In male MRL/*lpr* mice, the frequency of scratching increased slightly at 14 and 20 weeks of age (54.3 ± 14.1 and 108.3 ± 17.3, respectively).

3.2. Abrasion score in MRL/lpr mice

Dermal abrasions of the ear and rostral back were observed in male and female MRL/*lpr* mice ($n=6$) every 2 weeks from 8 to 20 weeks of age. The abrasion score in male and female mice did not change until 16 weeks of age (Fig. 2A). In the example shown, the female MRL/*lpr* mouse has an abrasion score of 6 (the sum of scores of 3 for the ear and 3 for the rostral back) (Fig. 2B).

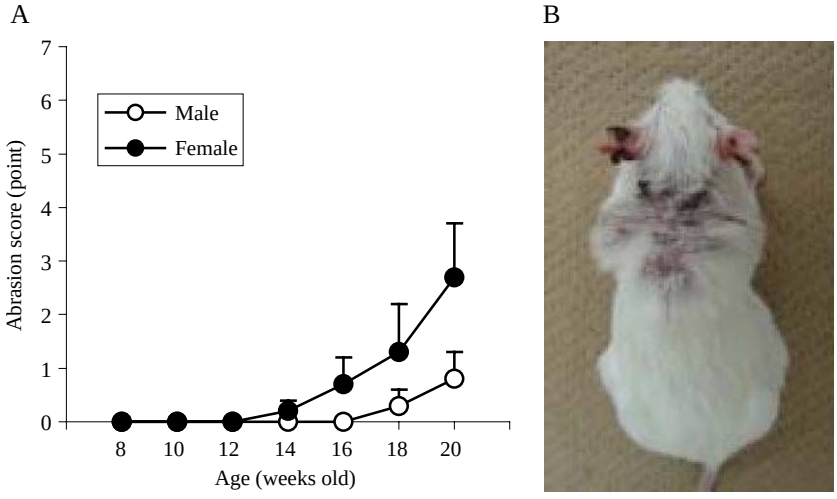

Fig. 2. Dermal abrasions of the ear and the rostral back in MRL/*lpr* mice. The dermal abrasions of the ear and the rostral back in male and female MRL/*lpr* mice (male; $n=6$ and female; $n=6$) was observed every 2 weeks from 8 to 20 weeks of age in a specific pathogen-free environment. The abrasion score was calculated as the total of a two-part score (ear plus rostral back) as follows: ear — 0, fine; 1, abrasion; 2, slight bleeding and abrasion; 3, scab and abrasion; 4, serious organization incrustation; rostral back — 1, good fur and normal skin; 2, slight alopecia and abrasion; 3, scab and abrasion. The maximum total score was 7. The ordinate represents the abrasion score (means and S.E.M.) in males (open circles) and females (closed circles) with aging (A). In the example shown, the female MRL/*lpr* mouse has an abrasion score of 6 (the sum of scores of 3 for the ear and 3 for the rostral back) (B).

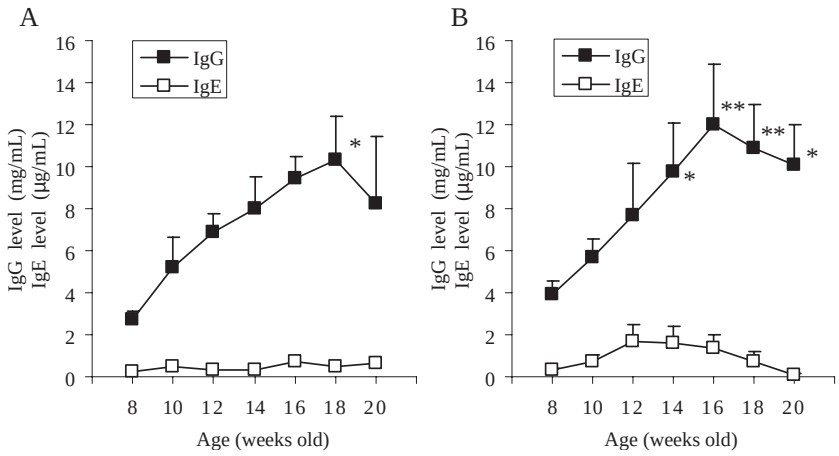

Fig. 3. IgE and IgG levels in MRL/*lpr* mice in a specific pathogen-free environment. Serum total-IgE and total-IgG levels in male and female MRL/*lpr* mice (male, $n=6$, and female, $n=6$) were measured every 2 weeks from 8 to 20 weeks of age. The ordinate represents the serum concentrations (means and S.E.M.) of IgE (open square) and IgG (closed square) in males (A) and females (B) with aging. $^{*}P<0.05$, $^{**}P<0.01$ when compared with the concentration of immunoglobulin at 8 weeks of age by multiple comparisons of time effects during longitudinal measurement designed.

3.3. Immunoglobulin levels with aging in MRL/lpr mice

The serum IgE levels in both male and female MRL/*lpr* mice ($n=6$) were near the limit of detection and did not significantly increase during the experimental period (Fig. 3A and B). On the other hand, in both male and female MRL/*lpr* mice, the serum IgG levels showed a remarkable increase from 14 to 20 weeks of age (Fig. 3A and B). In female mice 8 weeks of age, IgG levels were 3.9±0.7 mg/ml, at 20 weeks this level increased to 10.1±1.9 mg/ml.

3.4. Immunoglobulin levels in MRL/lpr mice compared with those in NC/Nga and ICR mice

We compared the levels of IgE, IgG and immunoglobulin subtypes such as G_1 and G_{2a} in MRL/*lpr* mice ($n=8$) with those in NC/Nga ($n=8$) and ICR mice ($n=3$) at 20 weeks of age (Table 1). Serum IgE levels in MRL/*lpr* mice and ICR mice were very low at 20 weeks of age. On the other hand, NC/Nga mice had significantly elevated serum IgE levels (36.5±3.8 μg/ml). The serum IgG levels in both MRL/*lpr* and NC/Nga mice were significantly higher than those in ICR mice. In particular, the proportion of IgG_{2a} was much higher in MRL/*lpr* mice when compared to NC/Nga. In contrast, the proportion of IgG_1 was lower in MRL/*lpr* mice when compared to NC/Nga mice.

3.5. Evaluation of antipruritic activity of agents on the MRL/lpr mice

The antipruritic activity of chlorpheniramine, naltrexone and nalfurafine was tested on female MRL/*lpr* mice at 18–20 weeks of age. Chlorpheniramine was administered orally at 3 mg/kg and dose escalated up to 30 mg/kg ($n=8$). Even at maximum dosages, chlorpheniramine did not inhibit scratching behavior, and no significant difference was observed among the groups (Fig. 4A). In contrast, subcutaneous administration of naltrexone (3 mg/kg, $n=8$) reduced the scratching behavior significantly ($P<0.01$) (Fig. 4B). The oral administration of nalfurafine significantly decreased the frequency of scratching in a dose-dependent manner ($P<0.05$ and $P<0.01$ at doses of 30 and 100 μg/kg, respectively) (Fig. 4C).

4. Discussion

Our study has demonstrated that MRL/*lpr* mice, an animal model of autoimmune disease (Hamano et al., 1993; Okamoto et al., 2004), develop spontaneous and persistent scratching behavior. This behavior may serve as an animal model of pruritus in autoimmune disease based on the following four characteristics. First, the scratching behavior increased in frequency as the mice got older and

Table 1
Comparison of serum levels of IgE and IgG among three line mouse strains

Mouse Strain	Serum level of immunoglobulin subtypes				Proportion (%)		
	IgE (μg/ml)	IgG (mg/ml)	IgG_1 (mg/ml)	IgG_{2a} (mg/ml)	G_1/G	G_{2a}/G	G_1/G_2
MRL/*lpr*	0.8±0.2	1.61±3.3	7.6±1.8	5.6±0.5	47.1±5.1	40.8±4.7	133.4±26.3
NC/Nga	36.5±3.8[b]	46.3±3.0[b]	26.9±3.0[b]	3.8±0.3[b]	59.5±2.4[a]	8.8±0.9[b]	699.2±47.3[b]
ICR	0.6±0.0	0.9±0.1	0.5±0.1	0.4±0.0	52.7±5.7	47.3±5.7	118.3±28.9

Serum levels of total IgE, total IgG, total IgG_1 and total IgG_{2a} in MRL/*lpr* mice ($n=8$) were compared with those in NC/Nga ($n=8$) and ICR mice ($n=3$) at 20 weeks of age. [a]$P<0.05$, [b]$P<0.01$ for comparison of the immunoglobulin levels between MRL/*lpr* and NC/Nga mice by unpaired *t*-test or Welch test.

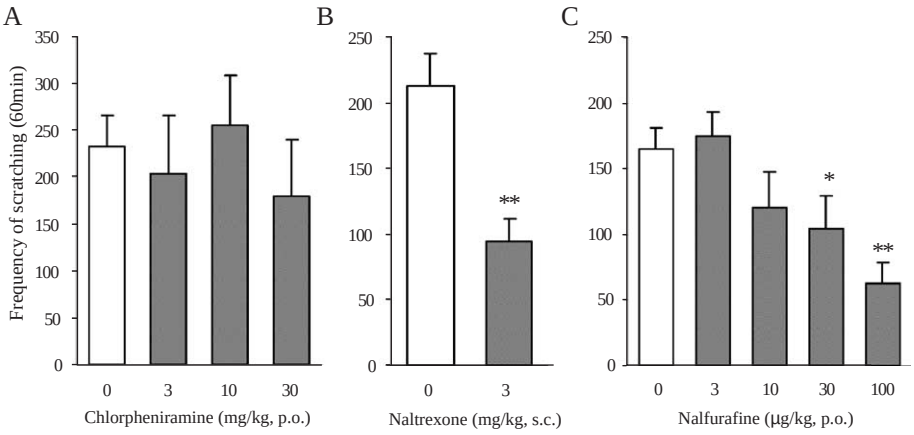

Fig. 4. Effects of the chlorphenniramine, naltrexone and nalfurafine on the spontaneous scratching behavior in MRL/*lpr* mice in a specific pathogen-free environment. Female MRL/*lpr* mice were evaluated at 18–20 weeks of age, each agent was administered to the same mouse and the dose was serially increased in the following manner. Day 1 (vehicle treatment): the vehicle was administered and the frequency of scratching behavior during 60 min was measured as baseline. From day 2 to day 5: the test compound, nalfurafine (3, 10, 30 and 100 μg/kg, p.o. −30 min, $n=8$), naltrexone (3 mg/kg, s.c. −30 min, $n=8$) or chlorpheniramine (3, 10, 30 mg/kg, p.o. −60 min, $n=8$) was administered before the recording and scratching behavior was quantified for 60 min. The ordinate represents the frequency of scratching for 60 min (means and S.E.M.). $^{*}P<0.05$, $^{**}P<0.01$. Statistical analysis was carried out using a paired *t*-test (B) or multiple comparisons of time effects for the longitudinal measurement (A and C) compared with the value at Day 1.

similar to other autoimmune phenomenon, was found more often in female mice compared to male mice (Fig. 1) (Lahita, 1997; Cooper and Stroehla, 2003). Second, in contrast to other animal models of pruritus (Matsuda et al., 1997), the spontaneous scratching behavior in MRL/*lpr* mice developed in a specific pathogen-free environment. This suggests that the scratching behavior in MRL/*lpr* mice is triggered by autoimmune factors rather than environmental factors. Third, the abrasions occurred after the scratching behavior was first observed (Fig. 2A) and were localized in areas that could be reached by the hind paws of the mice; suggesting these abrasions were the result of their scratching behavior (Fig. 2B). Finally the immunological features of the MRL/*lpr* mice differed significantly from other animal models of pruritus. NC/Nga mice, an animal model of atopic dermatitis, had elevated serum IgE levels which are known to correlate with their scratching behavior (Yamaguchi et al., 2001). In contrast, MRL/*lpr* mice had normal IgE levels and elevated total IgG levels (Fig. 3) which appear to correlate with their scratching behavior. IgG is primarily involved in delayed allergy and immunologic effects, recent studies have also shown that it may play a role in pruritus by the direct activation of primary sensory neurons through Fc gamma receptor I (FcγRI) (Andoh and Kuraishi, 2004). Thus the scratching behavior in MRL/*lpr* mice may result from this increase in serum IgG levels. Serum IgG subclasses also differed between NC/Nga mice and MRL/*lpr* mice (Table 1). NC/Nga mice had a high concentration and proportion of serum IgG_1 consistent with the predominantly Th2 type immunological response in atopic dermatitis (Matsuda et al., 1997). By contrast, neither the concentration nor proportion of serum IgG_1 in MRL/*lpr* mice was high, confirming a Th1 predominance in MRL/*lpr* mice (Takahashi et al., 1996). The immunological background, female predominance, development of this behavior in a specific pathogen-free environment and the development of skin abrasions following observation of this behavior suggest that the scratching behavior in MRL/*lpr* mice is an appropriate model of pruritus that occurs in autoimmune diseases.

Using this animal model of pruritus in autoimmune disease, we performed experiments to test the antipruritic effects of chlorpheniramine, naltrexone, and a novel κ-opioid receptor agonist, nalfurafine. The spontaneous scratching behavior in female MRL/*lpr* mice was determined to be antihistamine-resistant based on inefficacy of chlorpheniramine. This feature is concordant with what is observed in the pruritus associated with autoimmune diseases (Bergasa, 2003). On the other hand, an opioid receptor antagonist naltrexone, which has reported effectiveness to pruritus in primary biliary cirrhosis, significantly inhibited the scratching behavior. The effect of opioid receptor antagonists was also confirmed by pruritogen-induced scratching animal model (Maekawa et al., 2002). These findings suggest that the spontaneous scratching of the MRL/*lpr* mice is a response associated with pruritus rather than pain. Moreover, a novel κ-opioid receptor agonist nalfurafine was effective to the antihistamine-resistant and opioid antagonist-sensitive scratching behavior, indicating the possibility of antipruritic effect in autoimmune diseases.

Pruritus is a common symptom in autoimmune diseases, especially primary biliary cirrhosis where it has been reported to occur in 18.9% to 55% of patients (Talwalkar et al., 2003; Prince et al., 2002) and is the presenting symptom in almost one half of patients (Botero, 1978). The treatment of pruritus in primary biliary cirrhosis is limited and only a few patients benefit from antihistamines (Levy and Lindor, 2003). The severity and lack of

therapeutic options has led the American Association for the Study of Liver Diseases to propose uncontrolled pruritus in primary biliary cirrhosis as an indication for liver transplantation (Heathcote, 2000), stressing the need for the development of antipruritic agents that are beneficial for patients with autoimmune disease associated pruritus. Aged MRL/*lpr* mice have been reported to have primary biliary cirrhosis-like portal inflammation with cholangitis of small intrahepatic bile ducts, inflammatory cells, epithelioid granuloma, bile duct loss and antimitochondrial antibodies (Tsuneyama et al., 2001; Ohba et al., 2002; Leung et al., 1997), suggesting that the scratching behavior in MRL/*lpr* mice may mimic the pruritus in primary biliary cirrhosis and it will be one approach to contribute to this study.

The etiology of pruritus in autoimmune disease is unknown but one hypothesis is that it may be due to increased neurotransmission or neuromodulation of endogenous opioid peptides in the central nervous system (Bergasa, 2003; Zylicz and Krajnik, 1999; Thornton and Losowsky, 1988; Jones and Bergasa, 1999). Evidence for this has been the efficacy of opioid receptor antagonists in clinical trials of patients with pruritus associated with autoimmune disease (Bergasa et al., 1992; Wolfhnagen et al., 1997; Terg et al., 2002). These agents have not gained widespread acceptance due to a number of side effects, including an opiate-withdrawal-like syndrome. κ-Opioid receptor agonists and opioid receptor antagonists have similar actions, but novel κ-opioid receptor agonist nalfurafine has less adverse effects (Tsuji et al., 2001). In addition, nalfurafine has antipruritic effects at lower doses than its analgesic effects, suggesting that nalfurafine would be safe and effective against the pruritus in autoimmune diseases. Further clinical studies are needed to verify the efficacy and safety of nalfurafine for clinical usage.

In conclusion, spontaneous scratching behavior was observed in aged female MRL/*lpr* mice with features similar to the pruritus found in autoimmune diseases, indicating that it may be a useful animal model for this symptom. A novel κ-opioid receptor agonist, nalfurafine, inhibited this antihistamine-resistant scratching behavior and thus may have therapeutic value for pruritus in autoimmune diseases.

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