

***Mycobacterium vaccae* Reduces Scratching Behavior but not the Rash in NC Mice with Eczema: A Randomized, Blinded, Placebo-Controlled Trial**

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In a randomized, double-blinded, placebo-controlled trial, we previously showed that intra-dermal administration of a killed *Mycobacterium vaccae* suspension to school-aged children with atopic dermatitis ameliorates their disease. We wished to test the hypothesis that *M. vaccae* may also prevent the development of eczema. As it was not possible to do this in children, we studied the NC/Nga eczema mouse model. Thirty NC/Nga mice were randomized into a blinded, placebo-controlled trial where they received either 0.1 or 0.01 mg of *M. vaccae* (SRP299) or placebo given subcutaneously at 1 and 8 wk of age. Clinical eczema scores, as well as scratching frequency using a digital videotape system were assessed during the 26-wk study. Digital scratch scores correlated with clinical severity ($p = 0.001$). Although there were no significant differences in age of onset or severity of the rash between the three study arms, mice injected with 0.1 mg but not 0.01 mg of SRP299 had significantly lower peak scratch frequencies than controls (Hazard ratio 0.2; 95% confidence interval 0.1–0.7; $p = 0.01$). We conclude that in this NC/Nga mouse model, SRP299 did have a beneficial effect in reducing pruritis, a major clinical symptom of eczema, although it does not prevent the rash from developing.

Key words: atopic dermatitis/clinical trial/mouse/*Mycobacterium vaccae*/scratching
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We have previously shown in a randomized, double-blinded, placebo-controlled trial that intra-dermal administration of a suspension of killed *Mycobacterium vaccae* to school-aged children with moderately severe atopic dermatitis ameliorates their disease (Arkwright and David, 2001). Based on this observation, we wished to test the hypothesis that *M. vaccae* might also prevent the development of atopic dermatitis. Administration of *M. vaccae* to atopy-prone newborn infants was not possible and we therefore studied the efficacy of a killed *M. vaccae* suspension (SRP299) in preventing the development of eczema in the NC/Nga mouse model. As pruritis and thus scratching is a central clinical feature of eczema, we not only assessed the clinical severity of the eczematous rash but also the scratching behavior of the mice using a recently validated digital videotape system (Orito *et al*, 2004).

Dermatitis of the NC mouse has many similarities to that of human atopic dermatitis and develops spontaneously around the time of weaning in conventionally housed mice, but not in those housed in pathogen-free environments (Vestergaard *et al*, 2000). Dry, pruritic, lichenified skin lesions develop on the face, ears, neck, and back. Histologically, the skin lesions are characterized by hyperkeratosis, acanthosis, and parakeratosis, with a dermal infiltration of lymphocytes, eosinophils, mast cells, and macrophages.

Serum IgE levels are high and IFN- γ and IL-4 are expressed by mast cells and lymphocytes. Th1 cytokines exacerbate the dermatitis (Matsumoto *et al*, 2001). Th2 lymphocytes are not essential as STAT6 NC knockout mice develop dermatitis of similar severity to controls (Yagi *et al*, 2002). Steroid and tacrolimus ointment ameliorates the dermatitis when applied topically (Hiroi *et al*, 1998) and subcutaneous administration of transforming growth factor- β 1 (TGF- β 1) also ameliorates the disease (Sumiyoshi *et al*, 2002). SRP299 has previously been shown to suppress airway eosinophilia when injected subcutaneously into BALB/c mice and this effect can be blocked using anti-TGF- β antibodies (Zuany-Amorin *et al*, 2002). If subcutaneous injection of SRP299 were able to increase the activity of TGF- β in the NC mouse, then it might also ameliorate the dermatitis in this model.

Results and Discussion

General characteristics of study population Ten mice were randomized to each of three different treatment arms. All mice had developed eczema by 26 wk. There were no significant differences in the distribution of males and females between the groups (placebo group: seven males, 0.01 mg SRP299 group: five males; 0.1 mg SRP299 group: four males) (χ^2 test; $p = 0.4$). In this study cohort, male mice had a significantly more severe clinical course in terms of an earlier age of onset (males 10 (8–13) wk vs females 18 (11–23) wk; $p = 0.01$) and a higher peak clinical severity

Abbreviations: CI, confidence interval; TGF- β 1, transforming growth factor- β 1

score (males 14 (10–17) wk vs females 6 (4–15) wk; $p = 0.01$), although peak scratch frequency was similar (males 38 (25–67) wk vs females 30 (22–62) wk; $p = 0.5$). There was also a significant trend for mice, which developed eczema at an early age (age of onset of eczema in weeks) to have a higher peak clinical severity score (Spearman's $\rho = -0.58$; $p < 0.001$). No adverse effects of the treatment were noted and none of the mice developed a local reaction. In the overall study population, at any point in time, there was a significant correlation between clinical eczema severity and scratch frequency as assessed using the SCLABA digital videotape system (Spearman's $\rho = 0.41$; $p < 0.001$) (Fig 1). The correlation between the peak clinical and peak scratch scores was, however, not significant (Spearman's $\rho = 0.14$; $p = 0.2$).

Effect of *M. vaccae* (SRP299) on eczema in NC/Nga mice

No significant differences between the three treatment groups as to the age of onset of dermatitis (Kruskal–Wallis test; $p = 0.5$) or peak clinical eczema severity ($p = 0.2$) were observed (Figs 2 and 3A). The ages at which the mice developed peak clinical severity and peak scratch score were also similar between the treatment groups ($p = 0.8$). But the median peak scratch frequency (inter-quartile range) was significantly lower in mice injected with 0.1 mg (30 (24–33); $p = 0.002$ (Mann–Whitney U test)) but not 0.01 mg of SRP299 (16 (10–76); $p = 0.2$) when compared with the control group (53 (43–77)) (Fig 3B). Multi-variant analysis showed that controlling for gender did not effect the above trends (Table I).

Discussion This study demonstrated that although administration of *M. vaccae* did not delay the onset or reduce the severity of the eczematous rash, it was associated with a significant reduction in peak scratching frequency in NC/Nga mice given the higher (0.1 mg), but not lower (0.01 mg) dose of *M. vaccae* when compared with the control. Using the 95% confidence intervals displayed in Table I, we can

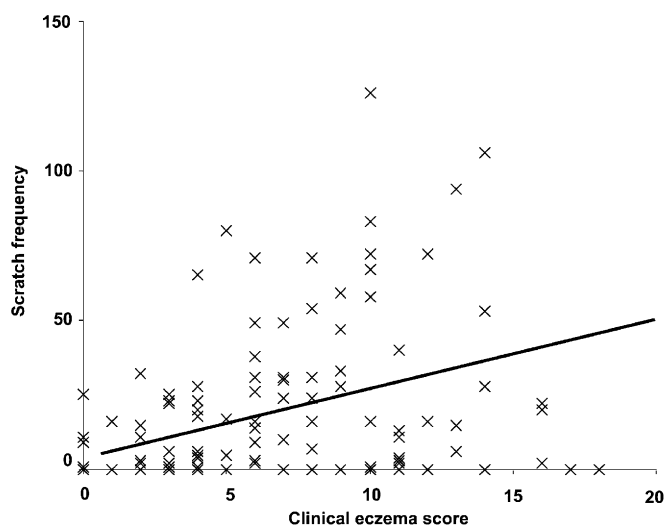


Figure 1
Scatterplot showing the correlation between clinical eczema score and scratch frequency during an 18-min observation period in NC/Nga mice. Simultaneous measurements of the two scores were made on individual mice between 8 and 26 wk of age. $N = 120$ measurements. Calculated linear regression line is shown.

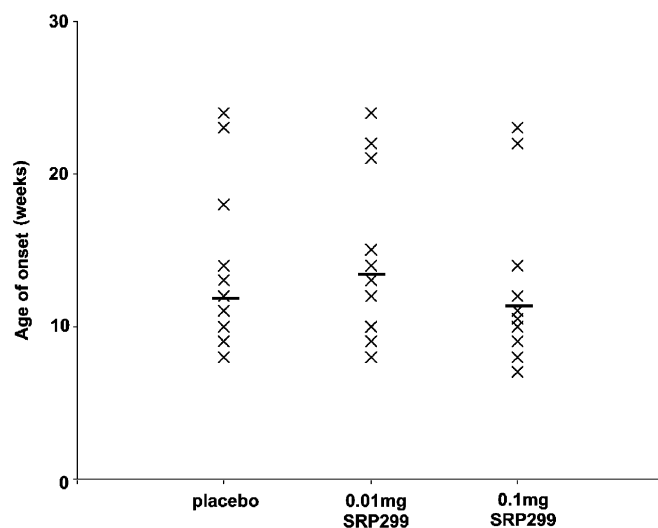


Figure 2
Comparative effects of placebo, SRP299 (0.01 mg dose), and SRP299 (0.1 mg dose) on the age of onset (weeks) of eczema (clinical eczema scores > 2) in NC/Nga mice. Bars represent median value.

be confident that the 0.1 mg dose of SRP299 will lead to a minimum of a 30% reduction in peak scratch frequency, whereas it will not decrease peak clinical eczema score by more than 10% or delay the onset of the eczema by more than 10%. A larger degree of variability is present in the 0.01

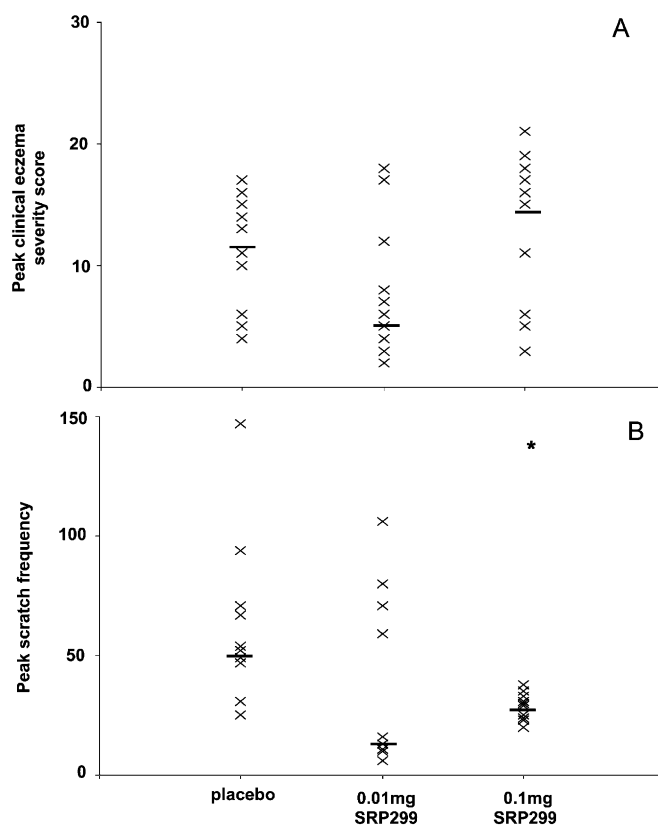


Figure 3
Comparative effects of placebo, SRP299 (0.01 mg dose), and SRP299 (0.1 mg dose) on the peak severity of eczema in NC/Nga mice. (A) Peak clinical eczema score, (B) peak scratch frequency. Bars represent median value. * $p < 0.05$ (Kruskal–Wallis H test).

Table I. Multi-variant analysis showing hazard ratio and 95% confidence interval (CI) for effects of "treatment with SRP299" and "gender" on the age of onset of eczema (weeks), peak clinical eczema severity, and peak scratch frequency

	Hazard ratio (95% CI)	p-value
Age of eczema onset		
0.01 mg SRP299 ^a	0.9 (0.4–2.1)	0.7
0.1 mg SRP299 ^a	0.4 (0.2–1.1)	0.1
Gender ^b	5.7 (2.1–15.4)	0.001
Peak clinical eczema severity		
0.01 mg SRP299 ^a	0.7 (0.3–1.9)	0.5
0.1 mg SRP299 ^a	2.7 (0.9–7.6)	0.1
Gender ^b	0.3 (0.1–0.7)	0.01
Peak scratch frequency		
0.01 mg SRP299 ^a	0.7 (0.3–1.9)	0.5
0.1 mg SRP299 ^a	0.2 (0.1–0.7)	0.01
Gender ^b	0.8 (0.4–1.8)	0.6

^aCox regression: compared with placebo group.

^bCox regression: females compared to males.

mg dose SRP299 results and thus further larger studies are required to support these data.

How does one explain the discrepancy between the observed improvement in pruritis/scratching behavior and the rash in these animals? Pruritis is the primary clinical problem in eczema (Stander and Steinhoff, 2002). It is the itch-provoked scratching that leads to secondary damage of the skin and thus the rash. The severity of the rash is not only determined by the underlying inflammatory eczematous process but is also influenced by confounding factors, particularly the degree of secondary bacterial infection. Measurement of scratching behavior is therefore likely to be a more direct and sensitive measure of the primary eczematous process than the rash. This may be particularly relevant to the study of laboratory animals because their housing, level of hygiene, and cleanliness are more likely to promote secondary bacterial infection of damaged skin, thus confounding the clinical picture even more so than with children.

We have previously shown that administration of *M. vaccae* to children with moderately severe eczema is associated with a significant improvement in school-aged children (Arkwright and David, 2001), but is no more effective than control in preschool children (Arkwright and David, 2003). If *M. vaccae* were also less efficacious in young mice, then assessment of a more direct and sensitive measure of the eczematous process, such as scratching behavior, might show a significant improvement, whereas no difference in the rash *per se* would be observed.

There will always be doubts as to the validity of animal models in the study of human disease. The clinical course, histology, and immunology suggest that the NC/Nga mouse is a useful model to study at least some aspects of human atopic dermatitis. The results of this study provide some evidence for a beneficial effect of *M. vaccae* even in young

animals. Testing this compound in young infants is the next step in order to determine whether this compound has a useful therapeutic place in this group of patients. The study of this NC/Nga mouse model has highlighted the fact that pruritis and thus scratching behavior need to be considered when assessing clinical eczema severity. Better measures of assessing this aspect of the disease in children are required if our understanding of the clinical aspects of this condition are to advance.

Materials and Methods

Mice Thirty NC/Nga mice housed in a non-specific pathogen-free environment (23°C ± 2°C, 60% ± 10% humidity, laboratory chow, and water *ad libitum*) were randomized into three equal groups of 10 mice to receive a subcutaneous injection of (1) 0.1 mg (10⁸ CFU) of *M. vaccae* (SRP299) (a gift from SR Pharma plc, London, UK), (2) 0.01 mg (10⁷ CFU) of SRP299, or (3) buffer alone in a volume of 0.1 mL at 1 wk and again at 8 wk of age. P. D. A. randomized the animals using a web-based random number generation program (www.graphpad.com/quickcalcs/randomize1.cfm). C. F., who was blinded to the treatment arm to which the mice had been randomized, made all clinical measurements as detailed below. Animal experiments complied with the standards in the guidelines of the University Animal Care and Use Committee of Tokyo University of Agriculture and Technology. All mice completed the study and were used in the analysis.

Assessment of eczema Clinical eczema scores were assessed using the method previously described (Matsuda *et al*, 1997). Briefly, the total clinical severity score was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (itch, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness). Scores were determined weekly from 5 until 26 wk of age. Scratching frequency was assessed at 6 weekly intervals between 8 and 26 wk. Briefly, the nape of the neck and hind limbs were marked with different colors and each mouse was placed in a transparent acrylic observation cage. Scratching frequency was recorded on a videotape for 18 min using a digital video camera (GR-DV700, JVC, Tokyo, Japan). Data were analyzed using the SCLABA system (Noveltec, Kobe, Japan) as previously described and validated in our laboratory (Orito *et al*, 2004). The primary outcome measures of the study were (1) age of onset of clinical eczema score >2, (2) peak clinical eczema score, and (3) peak scratch frequency score.

Statistical analysis As data in our study were not normally distributed (defined as mean/SE for skewness and kurtosis <2), results are quoted as median and inter-quartile range. The Mann-Whitney *U* test, Kruskal-Wallis test, and Spearman's correlation were used to examine trends in these non-normally distributed data. χ^2 analysis was used for binary data. Multi-variant analysis was performed using Cox regression. All statistics are quoted as results of two-tailed tests. The software package used was the SPSS 10.1 statistical package (Chicago, Illinois).

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