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Accumulation of immunoglobulin G against *Dermatophagoides farinae* tropomyosin in dorsal root ganglia of NC/Nga mice with atopic dermatitis-like symptoms

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ABSTRACT

Atopic dermatitis (AD), a chronic inflammatory skin disease, manifests as intractable itch, but its underlying mechanisms are poorly understood. This study assessed the relationship between immunoglobulin G (IgG) and dorsal root ganglia (DRG) in NC/Nga mice, a model of AD that manifests AD-like symptoms including itch. Immunohistochemical analysis showed large amounts of IgG in DRG extracts of NC/Nga mice with AD-like dermatitis, with a large fraction of the IgG distributed in satellite glial cells of the DRG. Proteomic analysis showed that this IgG was reactive against tropomyosin of *Dermatophagoides farinae*. These findings indicate that the accumulation of anti-tropomyosin IgG in DRG of atopic NC/Nga mice may be associated with the pathogenesis of AD-like symptoms, including itch.

1. Introductions

Atopic dermatitis (AD) is a skin disease involving eczematous skin lesions and chronic and intense pruritus. Clinically, itch in AD patients is resistant to conventional treatments, such as histamine H_1 receptor antagonists [1] [2], and such itch is called intractable itch [3]. The itch induces scratching behavior, resulting in greater damage to the skin barrier. This disruption of skin barrier leads to further itch, causing a vicious cycle called the itch-scratch cycle [4]. During this cycle, even painful stimuli evoke itch in AD patients [5]. This intractable itch lowers the quality of life in AD patients. Determining the fundamental mechanisms underlying intractable itch is therefore important in developing antipruritic treatments.

Cutaneous sensations, such as itch, pain and thermoception, are transmitted by primary afferent sensory neurons with cell bodies of dorsal root ganglia (DRG) and trigeminal ganglia [6]. DRG neurons with unmyelinated axons innervate the skin and convey pruritogenic stimuli to the brain. Moreover, itch-related neuropeptides and receptors are expressed in DRG during acute itch [7] [8]. Relatively few studies, however, have analyzed the cellular and molecular mechanisms in the nervous and immune systems that drive chronic itch.

The formation of IgG-ragweed pollen complexes has been reported to induce Ca^{2+} influx in cultured DRGs, with these IgG-

Abbreviations: AD, atopic dermatitis; DRG, dorsal root ganglia; GLAST, glutamate aspartate transporter; SGCs, satellite glial cells.

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antigen complexes inducing DRG neurons to release the itch-related peptide, substance P [9]. Ragweed pollen was found to induce itch-associated scratching responses in mice immunized with ragweed pollen, suggesting that the formation of IgG-antigen complexes on sensory neurons and/or nerve fibers may cause itch-related scratching behavior. To date, however, IgG reactive against specific antigen(s) has not been reported to accumulate *in vivo* in DRGs of humans or animals with pruritic skin diseases such as AD. This study therefore examined the relationship between IgG and DRGs in NC/Nga mice, an animal model of AD characterized by AD-like symptoms including itch. We found that IgG that accumulated in the DRG of atopic NC/Nga mice is directed against a tropomyosin of *Dermatophagoides farinae*.

2. Materials and methods

2.1. Animals

Male NC/Nga mice aged 9–10 weeks were purchased from Japan SLC (Shizuoka, Japan). These were divided into 2 groups. The control group (n = 15) was housed under specific pathogen-free condition and showed no AD-like symptoms, whereas the AD group (n = 15) was housed under conventional condition, resulting in the induction of AD-like symptoms. All mice were maintained in the experimental animal facility of Juntendo University Graduate School of Sports and Health Science under a 12-hr light: 12-hr dark cycle at a regulated temperature of 22–24 °C, with food and tap water provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee at Juntendo University Graduate School of Medicine and Graduate School of Sports and Health Science and conformed to the guidelines for the use of laboratory animals of the National Institutes of Health.

2.2. Preparation of murine DRG

The mice were anesthetized with somnopentyl (60 mL/kg, Kyoritu Seiyaku, Tokyo, Japan) and perfused transcardially with 10 mL phosphate-buffered saline (PBS). Upper to mid cervical DRGs were extracted and immediately frozen in liquid nitrogen.

2.3. Proteomic analysis

DRGs taken from three mice of the same group were pooled, homogenized in cell lysis buffer containing 9 M urea, 40 mM Tris and 4% CHAPS, and centrifuged at 15,000×*g* for 5 min. The protein concentration of each supernatant was determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA, USA). A 20 μg aliquot of each extracted protein sample was loaded onto an SDS-PAGE gel; following electrophoresis, the proteins were subjected to western blotting. A prestained marker (Novex Sharp Pre-Stained Protein Standard, Invitrogen, USA) for molecular weights was included on each gel. The membrane was blocked with 1% bovine serum albumin (BSA) in PBS-T (PBS containing 0.1% Tween 20) and then treated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:5000 dilution, Sigma, St, Louis, MO, USA) in blocking solution (BSA/PBS-T) for 90 min at room temperature. The chemiluminescence of each membrane was detected using Immunstar AP substrate (Bio-Rad, CA, USA) with a cooled CCD gel image analyzer (ATTO Co., Tokyo, Japan).

For re-blotting with antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology Inc., CA, USA), the blotted membrane was washed with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 0.7% 2-mercaptoethanol) for 30 min at 45 °C, blocked with BSA/PBS-T and incubated with anti-GAPDH antibody (1:500 dilution) in BSA/PBS-T at 4 °C overnight. After washing, the membrane was incubated with AP-conjugated goat anti mouse IgG antibody (1:5000 dilution) for 90 min at room temperature.

For LC-ESI-MS/MS analysis, the proteins on the SDS-PAGE gel were stained with Coomassie Brilliant Blue (CBB) R, and the proteins corresponding to immunoreactive bands were digested with trypsin (Promega, Madison, WI, USA). The tryptic peptides were subjected to LC-ESI-MS/MS analysis using a Thermo Fisher Scientific LXQ mass spectrometer with a nano-liquid chromatography (AMR, Inc., Tokyo, Japan) [10]. In this study, the peptides were separated on a Zaplous α Pep-C18 column (AMR, Inc., Tokyo, Japan). The Swiss-Prot database was subsequently searched with the MASCOT search engine (Matrix Science, London, UK).

2.4. Isolation of IgG from DRG extracts

Proteins from pooled DRGs from three mice were extracted by radio immunoprecipitation assay (RIPA) buffer containing 1 M Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonie P-40 and complete EDTA-free protease inhibitor (Roche Applied Science, Pennsburg, Upper Bavaria, Germany). Subsequently, 60 μL of Protein G-Sepharose 4 Fast Flow (GE Healthcare, CA, USA) resin were suspended in 1 mL RIPA buffer containing 20 μg of proteins extracted from DRGs and rotated at room temperature for 2 h. The resin was centrifuged at 700×*g* for 1 min and washed with RIPA buffer three times. Sample was eluted from the resin with SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 8.5, 10% glycerol, 2% SDS, 0.025% bromophenol blue, 0.1 M dithioerythritol) and incubated at 90 °C for 3 min. For LC-ESI-MS/MS analysis, five samples, each containing 20 μg DRG extracts from three mice, were combined and analyzed.

2.5. Immunohistochemistry

Upper to mid cervical DRGs taken from mice were immediately immersed in 4% paraformaldehyde in PBS at 4 °C for 4 h. After washing with PBS, pH 7.4, the DRGs were immersed in PBS containing 20% sucrose overnight at 4 °C. The tissue sections were embedded in optimal cutting temperature compound (Sakura Finetechnical co., Ltd., Tokyo, Japan) and quickly frozen on dry ice. Cryosections (thickness, 6 μm) were cut using a CM1520 cryostat (Leica, Nussloch, Germany). These sections were subjected to double-immunofluorescence staining, using Alexa Fluor 488-conjugated-donkey anti-mouse IgG antibody (Molecular Probes, OR, USA) or Alexa Fluor 594-conjugated-donkey anti-mouse IgG antibody (Jackson Immunno Research Laboratories, PA, USA), goat anti-peripherin antibody (Santa Cruz Biotechnology Inc., CA, USA), or rabbit anti-glutamate aspartate transporter (GLAST) antibody (Novus Biochemicals, MA, USA).

Briefly, the sections were washed twice with PBS containing 0.05% Tween-20 and blocked by incubation in PBS containing 2% BSA; 5% normal donkey serum containing 0.2% Triton X-100 (for anti-peripherin antibody) or containing 3% normal donkey serum, 3% goat serum and 0.2% Triton X-100 (for anti-GLAST antibody). The sections were subsequently incubated with primary anti-peripherin (1:100 dilution) or anti-GLAST (1:200 dilution) antibody. After three washes, the sections were incubated with secondary antibodies including Alexa Fluor 488-conjugated-donkey anti-mouse IgG antibody (1:500 dilution) and Alexa Fluor 594 donkey anti-goat IgG antibody (1:500 dilution, Abcam, Cambridge, UK.) to detect peripherin, or Alexa Fluor 594-conjugated-donkey anti-mouse IgG antibody (1:500 dilution) and Alexa Fluor 488 goat anti-rabbit IgG antibody (1:500 dilution, Jackson ImmunnoResearch Laboratories, PA, USA) to detect GLAST. The sections were cover-slipped with Vectashield-mounting medium for fluorescence