



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.

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1. Introduction

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 H₁ 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 Ca²⁺ 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 $15,000\times g$ 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 μ g 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 Immunarstar 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 μ L of Protein G-Sepharose 4 Fast Flow (GE Healthcare, CA, USA) resin were suspended in 1 mL RIPA buffer containing 20 μ g of proteins extracted from DRGs and rotated at room temperature for 2 h. The resin was centrifuged at $700\times g$ 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 μ g 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 μ m) 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 Immuno 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 ImmunoResearch Laboratories, PA, USA) to detect GLAST. The sections were cover-slipped with Vectashield-mounting medium for fluorescence

with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA). DRGs were visualized using a LSM 710 laser-scanning microscope (Carl Zeiss, Jena, Germany) and the images were captured using LSM software ZEN 2011.

2.6. Murine plasma and DRG extracts containing antibodies to *Dermatophagoides farinae* (*D. farinae*) antigen

Whole blood was collected into syringes containing 2.6 U heparin sulfate and centrifuged at 1200×g for 30 min, and the supernatant was collected. DRG extracts were collected using the same method as for proteomic analysis, with RIPA being the homogenate buffer.

2.7. Preparation of protein extracts from *D. farinae* and antigen determination

Proteins in lyophilized *D. farinae* powder (Biostir Inc., Kobe, Japan) were extracted with RIPA buffer. To determine the antigens reacting with the IgG derived from DRGs of the AD-NC/Nga mice, samples containing 7 µg of extracted proteins were separated by SDS-PAGE and subjected to western blotting using plasma (1:66.7 dilution in BSA/PBS-T) or DRG extracts (1:6.67 dilution in BSA/PBS-T) from three control- or AD-NC/Nga mice. Fractions containing mouse IgG were detected using anti-mouse IgG antibody, as described in the section on proteomic analysis.

3. Results

3.1. Detection of IgGs in murine DRG extracts

To confirm the presence of IgG in DRG extracts from AD-NC/Nga mice, the protein extracts were subjected to western blotting. Several bands reactive with anti-mouse IgG antibody, including bands around 110 kDa (I), 55 kDa (II), and 25 kDa (III), were detected in protein extracts from DRGs of AD-NC/Nga mice (Fig. 1A).

The molecular weights of the late two bands correspond to those of heavy chain and light chain of IgG, respectively. Although the most intense band corresponded to a molecular weight of around 110 kDa, which is a little smaller than that of intact IgG (150 kDa) based on a comparison with prestained molecular marker, LC-ESI-MS/MS analysis confirmed that this band contained intact IgG (data not shown). In contrast, extremely weak bands were observed in extracts from DRGs of control mice. The intensity of the heavy chain band, when normalized to that for anti-GAPDH IgG, was significantly ($p < 0.05$) higher in the DRG of AD-NC/Nga than of control mice (Fig. 1B).

Following isolation of IgG from the DRGs using Protein G-Sepharose 4, we subjected these samples to combination analysis, by SDS-PAGE and western blotting with anti-mouse IgG. We observed an immunoreactive signal of around 55 kDa in the sample from AD-NC/Nga mice, but almost no signal in the sample from control mice (Fig. 1C). These protein bands were subsequently cut out of the gel and digested with trypsin. LC-ESI-MS/MS analysis successfully identified several subclasses of IgG in the DRG extracts of AD-NC/Nga mice (Table 1).

3.2. Localization of IgG in DRG of AD-NC/Nga mice

Because sensory neurons containing C-fibers mainly respond to pruritic stimuli [11], we assessed the localization of IgG in C-fiber-containing neurons. Anti-peripherin antibody was used as a marker for C-fiber neurons. C-fibers in DRG neurons from both control and AD-NC/Nga mice were positive for staining with anti-peripherin antibody (Fig. 2B, F). Anti-IgG staining was observed in cells surrounding DRG neurons in AD-NC/Nga (Fig. 2G, H, I), but not control (Fig. 2C and D) mice, with no significant overlap between anti-IgG and anti-peripherin staining in DRGs from AD-NC/Nga mice (Fig. 2H and I).

We next assessed the localization of IgG in satellite glial cells (SGCs) surrounding the DRGs. Anti-GLAST antibody was used as a marker for SGCs. Anti-GLAST staining was observed in SGCs (Fig. 3B,

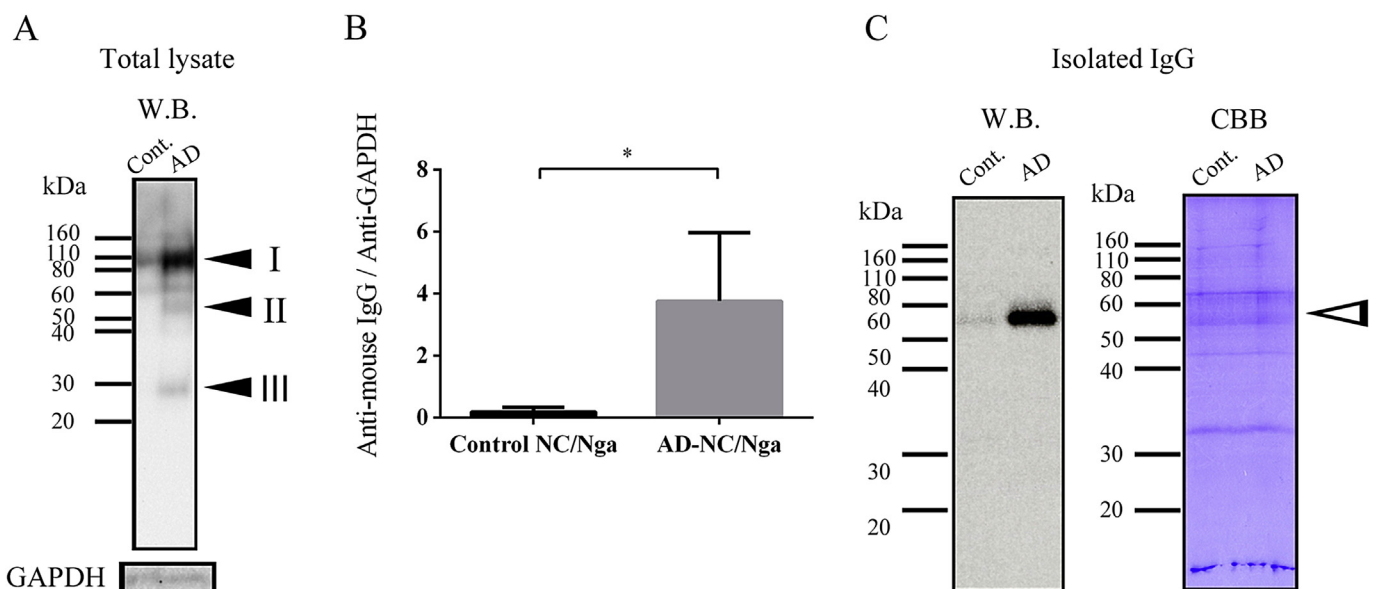


Fig. 1. Western blot analyses of mouse-IgG and GAPDH in DRG extracts.

(A) Western blotting of total extracts of DRGs with anti-mouse IgG. Filled arrowheads indicate the three major bands correspond to molecular weights of 110 kDa (intact IgG, I), 55 kDa (heavy chain, II), and 25 kDa (light chain, III), respectively. (B) Semi-quantitative analysis of band II indicated by a filled arrowhead in (A). The intensity of band II was normalized relative to that of GAPDH. * $p < 0.05$ by Student's *t*-test ($n = 5$). Values are expressed as means $\pm$ SD. (C) Detection of isolated IgG from DRGs with anti-mouse IgG and Coomassie Brilliant Blue staining. The white arrowhead indicates the band used for LC-ESI-MS/MS analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Identified peptide sequences from the band in Fig. 1(C).

Protein	Peptide sequences	Score*	Accession No.
Ig gamma-2A chain C region, A allele	184R.VVSALPIQHQQDWMSGK.E ²⁰¹	49	P01863
	227R.APQVYVLPPEEEMTKK.Q ²⁴⁵	52	
	275K.NTEPVLDSDGSYFYMSK.L ²⁹³	120	
Ig gamma-2B chain C region	136K.DVLMISLTPK.V ¹⁴⁷	54	P01867
	189R.VVSTLPIQHQQDWMSGK.E ²⁰⁶	72	
	210K.VNNKDLPSPIEP.T ²²³	44	
	232R.APQVYVLPPEEQLSR.K ²⁴⁹	49	
	280K.DTAPVLDSDGSYFYMSK.L ²⁹⁸	74	
Ig gamma-1 chain C region, membrane-bound form	199R.VNSAFAPIEK.T ²¹²	68	P01869
	221K.APQVYVLPPEE.E ²³³	38	
Ig heavy chain V region AC38 205, 12	74K.SSSATYMER.S ⁸⁵	39	P06330

* Individual scores >35 by MASCOT search indicate identity or extensive homology (p < 0.05).

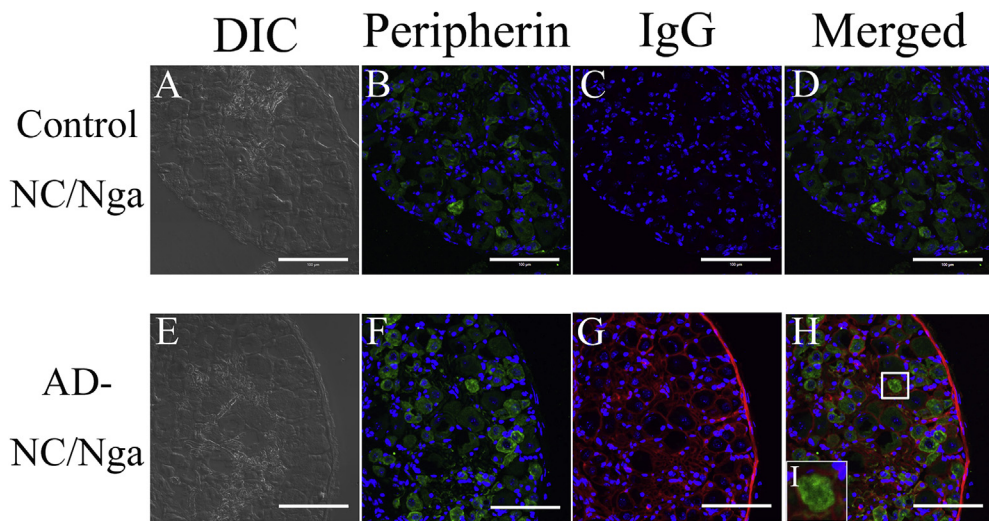


Fig. 2. Double immunofluorescence staining for mouse IgG and a marker of C-fiber neurons in DRGs. DRGs were visualized by differential interference contrast microscopy (A, E) and incubated with anti-peripherin (B, F) and anti-IgG (C, G) antibodies. D and H show the merged images of B and C and of F and G, respectively. I is an expanded image of the boxed area in the merged photograph (H). Scale bars, 100 μ m.

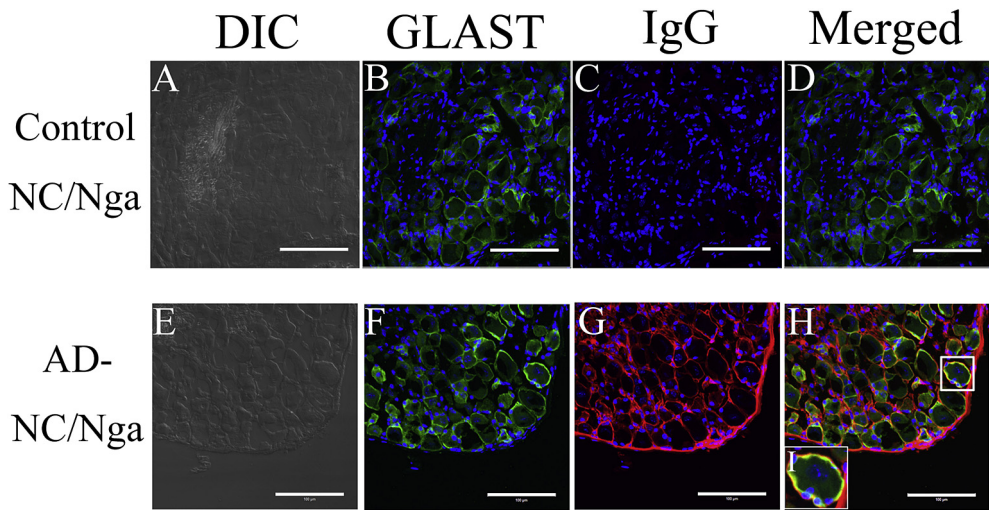


Fig. 3. Double immunofluorescence staining for mouse IgG and a marker of SGCs in DRGs. DRGs were visualized by differential interference contrast microscopy (A, E), and incubated with antibodies to GLAST (B, F) and IgG (C, G), respectively. D and H show the merged images of B and C and of F and G, respectively. I is an expanded image of the boxed area in the merged photograph (H). Scale bars, 100 μ m.

F) and overlapped with anti-IgG staining in DRGs from AD-NC/Nga mice (Fig. 3G, H, I). Almost no anti-IgG staining was detected in DRGs from control mice (Fig. 3C and D).

3.3. Identification of the antigen for the DRG-derived IgG

Because extracts of the mite, *D. farinae*, have been shown to cause AD-like symptoms in NC/Nga mice and are strong candidate antigens causing AD in humans, we analyzed extracts of *D. farinae* to identify the antigen against which the IgG in the DRGs of AD-NC/Nga mice was directed. Protein extracts of *D. farinae* were subjected to SDS-PAGE and western blotting with AD-NC/Nga plasma or IgG-containing DRG extracts as the first antibody and anti-IgG antibody as a second antibody. Plasma from AD-NC/Nga mice detected many immunoreactive signals against *D. farinae* extract (Fig. 4III), suggesting that many antigens in *D. farinae* extracts reacted with IgG in AD-NC/Nga mouse plasma. In contrast, little immunoreactivity was observed when blots of *D. farinae* extracts were incubated with plasma from control mice (Fig. 4I).

We next examined the immunoreactivity of protein extracts from DRGs with extracts of *D. farinae*. DRG extracts of AD-NC/Nga mice reacted with a single band, of around 35 kDa, in the extracts of *D. farinae* (Fig. 4IV), suggesting that a specific antigen in *D. farinae* reacted with the IgG in the DRGs of AD-NC/Nga mice. In contrast, no signal was detected when DRG extracts of control mice were reacted with *D. farinae* extracts (Fig. 4II).

To identify the specific 35 kDa antigen that reacted with IgG from DRG extracts of AD-NC/Nga mice, the gel band (Fig. 4V) was cut out, digested with trypsin, and analyzed by LC-ESI-MS/MS. This protein was identified as *D. farinae* tropomyosin, as it had a significant ion score ($P < 0.05$), and the highest protein score on a MASCOT search, with 79% sequence coverage of the sample from *D. farinae*. This result suggested that the analyzed band was tropomyosin.

4. Discussion

The present study showed that IgG accumulated in DRGs of AD-NC/Nga mice. Immunohistochemically, we showed that most of the IgG localized in SGCs surrounding the DRG neurons of AD-NC/Nga

mice, not of controls. Moreover, we found that the specific antigen for this IgG was *D. farinae* tropomyosin, suggesting that anti-tropomyosin IgG that accumulates in DRGs is associated with the pathogenesis of AD-like symptoms, including itch.

IgG-antigen complexes have been reported to contribute to the pathogenesis of chronic pain in immune-related diseases, in addition to inflammatory mediators and cytokines [12] [13]. Moreover, Fc-gamma receptor I (FcγRI), which is one of the receptor families binding to the Fc domain of IgG and the only high-affinity activating receptor, was found to be expressed in nociceptive DRG neurons [14]. Transient receptor potential canonical 3 (TRPC3) channels have been reported to be co-expressed, along with FcγRI, in the same DRG neurons, with these channels acting to stimulate IgG-antigen complex-induced Ca^{2+} response to pain [15]. Although itch and pain sensations share many overlapping mediators, receptors and nerve fibers [16], a few studies have assessed the specific role of IgG-antigen complexes in itch signaling. For example, when incubated with cultured DRG cells of mice immunized with ragweed pollen, IgG-antigen complexes increased the influx of Ca^{2+} ions and the release of substance P, suggesting that the formation of IgG-antigen complex on DRGs may cause itch-related scratching behavior [9]. In this study, we detected the presence of IgG in isolated DRGs of NC/Nga mice with AD-like symptoms. Application of *D. farinae* extracts to the skin of NC/Nga mice increased extract-specific serum IgG and IgE levels [17]. In addition, our western blotting analysis showed that the levels of IgG directed against many antigens in *D. farinae* extracts were higher in plasma of AD-NC/Nga than of control mice. Generally, IgE, not IgG, has been regarded as the most critical immunoglobulin class in pathophysiological allergic conditions such as AD and asthma [18]. However, LC-ESI-MS/MS analysis was unable to detect IgE in atopic DRG extracts in this study (data not shown).

We found that a large fraction of the IgG found in atopic DRGs localized to the SGCs around the DRGs. Because there was almost no IgG immunoreactivity in the DRGs of control, the recruitment of IgG to the DRGs was associated with the development of AD. SGCs are known to ensheath tightly the soma of sensory neurons and to facilitate bidirectional regulation of SGC function and neuronal excitability. Moreover, SGCs have been reported to contribute to pain modulation [19–21] and to express certain pain-related

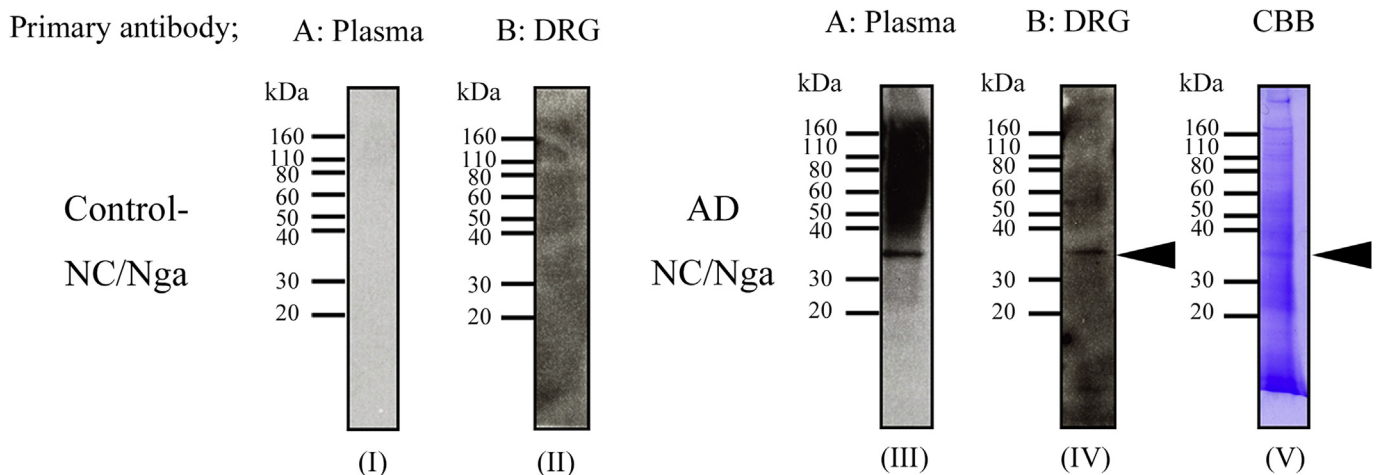


Fig. 4. Detection of antigens in *D. farinae* extracts by one-dimensional SDS-PAGE and western blotting with IgGs in NC/Nga mouse plasma or DRG extracts.

Proteins from freeze-dried *D. farinae* powder were applied to each lane (I ~ V). Following electrophoresis, the lanes were incubated with plasma (I) or DRG extracts (II) from control NC/Nga mice, and with plasma (III) or DRG extracts (IV) from AD-NC/Nga mice. All lanes were subsequently incubated with anti-mouse IgG secondary antibody. Lane V shows Coomassie Brilliant Blue staining of the proteins extracted from *D. farinae*. The filled arrowhead in IV represents the position of an antigen to the mouse IgG. The filled arrowhead in V shows the protein band corresponding to the position of the band in IV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

receptors, such as transient receptor potential vanilloid 4 (TRPV4) and P2Y1 [22]. Although Fc γ RI may be present in glial cells of murine DRGs [9], no study to date has reported the involvement of SGCs in the mechanism of itch sensation. The functional role of IgG on SGCs of DRGs derived from AD-NC/Nga mice may be clarified by identifying the IgG specific receptor on SGCs.

We had hypothesized that IgG in DRGs of AD-model mice may react with *D. farinae* derived antigens. This study was the first to identify a specific antigen, *D. farinae* tropomyosin, in AD-NC/Nga mice. Tropomyosin is a constituent protein of muscle and a major allergen in AD [23] [24] and shellfish allergy [25].

In conclusion, these findings indicate that tropomyosin-specific IgG, which localizes mainly to SGCs, may be associated with the pathogenesis of AD-like symptoms, including itch.

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