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Clonal differences in IgE antibodies affect cutaneous anaphylaxis-associated thermal sensitivity in mice

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Abstract

Cellular and molecular mediators of immune responses are increasingly implicated in acute and chronic pain pathophysiologies. Here we demonstrate that passive cutaneous IgE/Ag anaphylaxis provokes increased thermal sensitivity in the hind paw tissue of mice. The murine anti-DNP IgE antibodies SPE-7 and ϵ 26 are known to induce differential cytokine production in bone marrow cultured mast cells *in vitro* without antigen challenge. We found a novel, antigen-dependent heterogeneity in the thermal pain responses elicited in the hind paws between SPE-7 and ϵ 26 sensitized DNP-challenged mice. Mice experienced pronounced hind paw thermal sensitivity lasting 6 hours after DNP challenge when sensitized with SPE-7 but not ϵ 26 IgE. The two IgE clones induced equivalent hind paw edema, neutrophil influx, cytokine production, and reduction in tissue histamine content *in vivo*, and bound to the same or overlapping epitopes on the DNP antigen *in vitro*. Therefore IgE antibodies against the same antigen can induce comparable inflammation, yet contribute to markedly different anaphylaxis-associated pain within an allergic response, suggesting that non-canonical IgE binding partners such as sensory neurons may play a role in allergy-related pain responses.

Keywords

Cytokinergic IgE; passive cutaneous anaphylaxis; thermal pain

1. Introduction

Allergens enter tissues at environmental interfaces such as the airway, skin and digestive tract, where they bind and cross-link complexes of Immunoglobulin E (IgE) bound to the high-affinity IgE receptor Fc ϵ R1 that is predominantly present on tissue-resident sentinel mast cells [1]. Within minutes after allergen exposure, mast cells become activated and

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degranulate, releasing a variety of mediators, including histamine, that regulate the symptoms of classic anaphylactic reactions [1,2]. The high affinity of monomeric IgE for the FcεR1 allows IgE-FcεR1 complexes to form on the surface of mast cells in the absence of antigen. This “sensitization” phase effectively primes the mast cells for rapid intracellular responses upon antigen encounter [3,4].

SPE-7 [5] and ε26 [6] are IgE antibodies that both recognize the hapten 2,4, dinitrophenyl (DNP). It has been shown that these IgEs alone, in the absence of antigen, can promote survival and activation of bone marrow cultured mast cells (BMCMCs), and stimulate their mediator production *in vitro* (reviewed in [7]). Kitaura et al. have shown that in the absence of DNP challenge, SPE-7 sensitization induces greater IL-6 production from BMCMCs compared to ε26 [8]. IgE antibodies have also been found to modulate airway inflammation independent of antibody-specificity [9] and elevated non-specific IgE in serum of atopic individuals suggests a role for total IgE in pathophysiology of atopy [10]. Therefore, different inflammatory outcomes mediated by different clones of IgEs that react to the same antigen are of considerable pathophysiological interest.

In addition to their well known regulatory functions in allergic and inflammatory diseases as well as host defense [1,2,11], mast cells are increasingly recognized as important mediators of acute and chronic pain in both clinical and experimental contexts [12,13]. While IgE/Ag-provoked pain has previously not been demonstrated in mice, Lavich et al. showed an IgE/Ag-induced transient thermal hyperalgesic response in the hind paws of rats [14]. IgE-dependent, mast cell-driven allergic contact hypersensitivity to the hapten oxazolone has also been shown to induce a persistent tactile sensitivity at the site of challenge [15].

We sought to characterize the contributions of the two “cytokinergic” [8] anti-DNP IgE antibodies – SPE-7 and ε26 – to changes in pain sensitivity associated with passive cutaneous anaphylaxis (PCA) reactions in the hind paws of male ND4 mice. Here we provide the first evidence in mice that local, passive IgE sensitization can elicit antigen-dependent changes in thermal pain sensitivity in the hind paws of mice. We also demonstrate a novel functional heterogeneity of IgE/Ag-induced pain outcomes in PCA reactions mediated by different clones against the same antigen *in vivo*. The intersection of allergies and pain is an area of growing interest with emerging epidemiological [16], clinical [17] and experimental [15,18] evidence that atopic signaling can drive changes in pain sensitivity through a variety of mechanisms. Our data indicate that the *nature* of the participating IgE molecules can also be a differentiating factor in anaphylaxis-associated pain in mice.

2. Materials and Methods

2.1. Animals

2–6 month old male ND4 Swiss mice (Harlan Laboratories, Indianapolis, IN) were housed in Macalester College’s animal facility, in accordance with National Institutes of Health-approved guidelines, with a 12-hour light/dark cycle and free access to food and water. ND4 Swiss mice were chosen for their well-established use in rodent pain models. Mice were age-matched within all experiments, and behavioral responses did not vary between

experiments using mice of different ages (Supplementary Figure 1). Macalester College's Institutional Animal Care and Use Committee approved all experimental procedures.

2.2. Drug administration

Hind paws of mice were passively sensitized with SPE-7 (Sigma Aldrich, St. Louis, MO) or ϵ 26 (Amgen, Thousand Oaks, CA; generous gift of Dr. Stephen Galli, Stanford University, Stanford, CA) clones of anti-DNP IgE dissolved in 0.9% saline (100ng in 10 μ l per paw) before systemic challenge (by tail vein or retro-orbital injection) at 24 hours post-sensitization with human serum albumin-conjugated DNP (DNP-HSA; referred to as DNP elsewhere in the text; Sigma Aldrich, Saint Louis, MO; 400 μ g in 200 μ l per mouse). DNP challenge was co-administered with 0.05% Evans blue dye (Sigma Aldrich, Saint Louis, MO) as a marker for plasma extravasation [19]. Control animals were either sham-sensitized or sham-challenged with 0.9% saline as indicated in the figure legends.

2.3. Measurement of thermal sensitivity

Mice were placed in a Plexiglas cylinder on a hotplate analgesia meter (Harvard Laboratories, Edenbridge, KY) maintained at $51.0 \pm 0.5^{\circ}\text{C}$ and removed when prolonged retraction, flipping/licking of the hind paw, or jumping with both hind paws off the hotplate was observed, but no longer than 40 seconds (after which tissue damage may occur), as previously described [20]. Two baseline hotplate latencies were taken 24 and 48 hours before the experiment. Changes in thermal latency were quantified by subtracting the mean baseline thermal latency from the experimental thermal latency at each time point for each mouse.

2.4. Hind paw edema

Change in hind paw width measured using digital calipers ($\pm 0.1\text{mm}$; VWR, Radnor, PA) was calculated as an average of the left and right paw widths. Baseline paw widths for each mouse were taken before treatment and these values subtracted from post-treatment paw widths to calculate tissue edema as previously described [20].

2.5. Histology

Excised hind paws were fixed in 10% buffered formalin for 24 hours, transferred to 70% ethanol, decalcified for 1–2 weeks in 15% EDTA, hydrated, and embedded in paraffin. 4 μ m sagittal sections were stained with Toluidine blue (pH 2.3 for 3 minutes) for quantification of mast cell degranulation status at 400x magnification on an Olympus CX21LED light microscope (Olympus Corporation of the Americas, Center Valley, PA) with 3 biological replicates per treatment. Toluidine blue-stained mast cells were counted in 10 fields/section. Degranulation was scored based on the number of granules observed outside the boundary of the cell: intact (0), mild (1–10), moderate (11–20), and extensive (20+) as previously described [20]. Researchers performing cell counts were blind to treatment.

2.6. Measurement of myeloperoxidase activity

Excised footpads were preserved and processed as previously described [20] to measure myeloperoxidase activity as a marker for neutrophil infiltration into the hind paw tissue. Optical density (OD) was normalized to tissue weight and represented as OD/g of wet tissue.

2.7. Gene expression

Excised footpads were flash frozen in liquid nitrogen, stored at -80°C , homogenized in RB buffer (Midwest Scientific, St. Louis, MO) with a Tissue Tearor, and centrifuged at 2000 rpm for 4 minutes. Total RNA was extracted from tissue homogenate using the Total RNA Mini Kit (Midwest Scientific, St. Louis, MO) according to manufacturer's instructions and the concentration was quantified using a Nanodrop ND-1000 Spectrophotometer (ThermoScientific, Wilmington, DE). Total RNA was reverse transcribed using the Superscript III First Strand Synthesis System (Life Technologies, Carlsbad, CA) in a 2720 Thermal Cycler (Life Technologies) and the relative transcript abundance of target genes determined using TaqMan Gene Expression Assays (Life Technologies) on a Bio-Rad iCycler (Bio-Rad, Hercules, CA) or an ABI StepOnePlus™ Real-Time PCR System (Life Technologies). Target genes included *interleukin-1 β* (*IL-1 β* ; Mm00434228_m1), *interleukin-6* (*IL-6*; Mm00446190_m1), and *interleukin-10* (*IL-10*; Mm00439614_m1). Fold expression was calculated using the $\Delta\Delta\text{Ct}$ method [21] using the housekeeping gene β -2-microglobulin (*β 2m*; Mm00437762_m1) and untreated footpad tissue as normalizing controls).

2.8. Measurement of histamine and cytokines

Excised hind paws were flash frozen in liquid nitrogen and homogenized in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor (Cocktail Set I; EMD Millipore, Billerica, MA) using a Tissue Tearor (BioSpec; Model 985370). Homogenates were incubated on ice for 20 minutes, centrifuged at 2000 rpm for 10 minutes at 4°C , and lysates stored at -80°C . Histamine (Neogen Corporation, Lansing, MI), and cytokines IL-1 β , IL-6, and IL-10 (R&D Systems, Minneapolis, MN) were quantified using ELISA according to manufacturers' instructions. Cytokine concentrations were normalized to the total protein concentration in each sample using the DC Protein Assay Kit I (Bio-Rad, Hercules, CA). All absorbances were recorded using a PowerWave XS microplate spectrophotometer (BioTek Instruments, Winooski, VT).

2.9. Epitope comparison ELISA

Anti-DNP ϵ 26 was biotinylated using ChromaLink Biotin Antibody Labeling Kit (Solulink, Inc., San Diego, CA). ELISA/EIA half-area plates (Corning Incorporated, Corning, NY) were coated with DNP (0.25 μg /well; 50 μl) overnight, incubated with 1.0 μg /ml to 0.002 μg /ml of biotinylated ϵ 26 in PBS for 2 hours, exposed to Streptavidin-HRP (50 μl /well; 20 min; R&D Systems, Minneapolis, MN) and tetramethylbenzidine substrate mixed 1:1 with hydrogen peroxide (50 μl /well; 20 min; R&D Systems, Minneapolis, MN). 25 μl of 2N H_2SO_4 (Sigma Aldrich, St. Louis, MO) were added to stop the reaction. Binding was assessed using color change; corrected absorbance values ($\text{OD}_{450\text{ nm}} - \text{OD}_{570\text{ nm}}$) were used to establish a standard curve of biotinylated ϵ 26 IgE binding to DNP, and determine the

saturating concentration (1.0µg/ml) of ε26-biotin to DNP. To measure competitive binding, DNP-coated plates were first incubated with non-biotinylated SPE-7 (83µg/ml to 0.12µg/ml in PBS) or ε26 IgE (256µg/ml to 0.28µg/ml in PBS) for 2 hours, re-incubated with 1.0µg/ml of biotinylated ε26 (50µl/well) for 2 hours. Binding was assessed as above. All incubations were performed at room temperature, and followed by 3 washes in 0.05% Tween 20 in PBS.

Maximum binding (B_0) was defined as the average absorbance of wells incubated with biotinylated ε26 IgE in the absence of any non-biotinylated IgE. Blocking efficiency was determined as the percentage of absorbance of biotinylated ε26 IgE bound over B_0 . All absorbances were recorded using a PowerWave XS microplate spectrophotometer (BioTek Instruments, Winooski, VT). Non-linear curve fitting was applied to the absorbance data in Prism 5.0 (GraphPad, San Diego, CA) using the exponential decay formula $y(t) = a * e^{Kt}$. Best-fit values for K within the 95% confidence interval were compared between ε26 and SPE-7 competitive binding curves.

2.10. Statistical analysis

Data were processed using Microsoft Excel and graphed with Prism 5.0 (GraphPad, San Diego, CA). Statistical analyses were performed using JMP 10.0 (SAS, Cary, NC) unless otherwise indicated. All data are presented as the mean ± SEM, analyzed by Student's *t*-test or one-way ANOVA with the Tukey-Kramer HSD *post hoc* test at each time point. Statistical significance was defined as $p < 0.05$, and denoted in the figure legends as: $p < 0.05$ [*], $p < 0.01$ [**], $p < 0.001$ [***], $p < 0.0001$ [****], $p < 0.00001$ [*****].

3. Results and discussion

We have previously shown that mast cell degranulation by administration of basic secretagogue compound 48/80 (c48/80) can directly induce thermal and mechanical sensitivity in the hind paw of ND4 and C57BL/6 mice. This sensitivity is abrogated in mast cell-deficient C57BL/6-*Kit^{W-sh/W-sh}* mice and is restored by plantar reconstitution with bone marrow cultured mast cells [20]. Classic allergenic activation of mast cells by IgE/Ag cross-linking has not been previously linked with changes in pain sensitivities in mice. Lavich et al. showed that IgE/Ag challenge in the hind paws of rats is associated with a transient change in thermal sensitivity to a vertically applied punctate heat source, and that this change is synergistically regulated by histamine, serotonin and bradykinin, but did not implicate mast cells as the cellular regulators of this change in pain behaviors [14].

3.1. Systemic DNP challenge following SPE-7 but not ε26 sensitization provokes antigen-dependent thermal sensitivity in the hind paws of mice

We found that systemic DNP challenge in male ND4 mice sensitized 24 hours earlier with the SPE-7 clone of anti-DNP IgE induced a pronounced thermal sensitivity (8–10 second swifter paw withdrawal from a 51°C hotplate) for up to 6 hours after antigen challenge (Figure 1A); pain responses were no longer detectable by 8 hours (data not shown). In contrast, mice sensitized with the ε26 clone of anti-DNP IgE did not show a significant increase in thermal sensitivity. Paw withdrawal behaviors of ε26-sensitized and DNP-challenged mice remained indistinguishable from sham-sensitized mice challenged with

DNP (Figure 1A). SPE-7 induced an antigen-dependent change in thermal sensitivity; neither SPE-7 nor ϵ 26-sensitized mice showed increased thermal sensitivity when sham-challenged with saline (Figure 1A).

As both ϵ 26 and SPE-7 have been characterized as “cytokinergic” IgEs capable of mast cell activation *in vitro* in the absence of antigen [8], we assessed thermal responses in mice on the day of sensitization prior to antigen challenge and found that neither SPE-7 nor ϵ 26-sensitized mice showed any changes in thermal sensitivity compared to sham-sensitized mice in the 24 hours between sensitization and antigen challenge (Figure 1B). Bax and Gould have recently reviewed the conformational isomerism of SPE-7 that allows it to bind structurally unrelated antigens [7]. However, even if SPE-7 can potentially bind non-DNP targets *in vivo*, such binding does not appear to contribute to differential thermal sensitivity, as the latter responses are strictly DNP-dependent.

Skin swelling is a primary indicator of acute, cutaneous, IgE-dependent allergic inflammation [19]. However, while SPE-7 and ϵ 26 anti-DNP IgEs provoked different pain behaviors, they induced equivalent, antigen-dependent hind paw edema. No swelling was detected in any sensitized mice when sham-challenged with saline at 24 hours post-sensitization (Figure 1C) or on the day of sensitization with either antibody clone (Figure 1D). We visually confirmed DNP-dependent tissue swelling by hind paw extravasation of systemically co-injected Evans Blue dye (Figure 1E). Sham-challenge following sensitization with either IgE clone did not produce detectable changes in dye extravasation, and IgE sensitization alone did not induce any visible changes in hind paw appearance (Figure 1E). As the commercial preparation of SPE-7 contains the preservative sodium azide, we further confirmed that sodium azide administration followed by systemic DNP treatment did not cause any decrease in paw withdrawal latencies or edema (Supplementary Figure 2).

Our findings indicate that IgE/Ag-driven PCA reactions can induce pain, and that the precise clonotype of the IgE antibodies involved may differentially regulate the amplitude and intensity of the pain response. We replicated our findings of increased pain sensitivity in SPE-7/DNP but not ϵ 26/DNP reactions in a second set of experiments testing mechanical sensitivity to pressure applied to the hind paw tissue. We found that SPE-7/DNP-treated mice showed a marked increase in hind paw sensitivity to pressure 1–6 hours after DNP challenge while ϵ 26/DNP-treated mice did not (Supplementary Figure 3).

3.2. ϵ 26 and SPE-7 sensitization followed by DNP challenge induce equivalent neutrophil influx and histamine release in the hind paw

Given our novel finding of IgE clonotype-driven functional heterogeneity in PCA-associated pain, we asked whether the differential thermal sensitivity in SPE-7 vs. ϵ 26-sensitized mice was associated with differences in neutrophil influx into the tissue following IgE/Ag-mediated mast cell activation. Mast cells regulate neutrophil entry into inflamed tissues [22,23], and neutrophils have been shown to be critical to the development of rat hind paw pain following ovalbumin sensitization and challenge [24]. Furthermore, blockade of neutrophils with fucoidan pre-treatment reduces mast cell secretagogue-provoked thermal sensitivity in mice [20], and carrageenan-induced pressure sensitivity in rats [25]. Here we

found that both SPE-7- and ϵ 26-sensitized DNP-challenged mice showed 2.5 to 3-fold increases in neutrophil influx as indicated by relative levels of myeloperoxidase (MPO) in the hind paw at 1 hour (Figure 2A, left). Increases in hind paw MPO levels were antigen dependent, as sham-challenged sensitized mice showed no such increases (Figure 2A, left). MPO levels in the tissue did not change within 6 hours of sensitization alone with either clone of antibody (data not shown), and after DNP challenge, MPO levels were indistinguishable from controls by 6 hours (Figure 2A, right).

To assess whether differential thermal sensitivities observed were associated with differences in degranulation, we used an established metric of mild, moderate and extensive degranulation states based on numbers of granules counted outside the cell boundary in hind paw tissue sections stained with Toluidine blue [20]. We found that DNP challenge invoked equivalent mild, moderate, and extensive degranulation in SPE-7 and ϵ 26-sensitized mice at 1 hour after DNP challenge (Figure 2B, left). Surprisingly, at 6 hours after DNP challenge, ϵ 26-sensitized mice showed an increase of ~1.5–2-fold in the proportion of moderately and extensively degranulated mast cells compared to SPE-7-sensitized mice (Figure 2B, right). To further compare the extent of degranulation following IgE/Ag reactions mediated by these two clones, we measured hind paw histamine levels by ELISA at 1 and 6 hours following DNP challenge. Enzymes such as histidine decarboxylase rapidly degrade histamine released in the tissue following mast cell degranulation [26], allowing the reduction in tissue histamine levels to be used as an index of total degranulation of resident mast cells [20]. Plantar histamine levels were significantly and equivalently reduced (~2-fold at 1 hour, ~1.5-fold at 6 hours) following DNP challenge in both SPE-7 and ϵ 26-sensitized mice at both time points (Figure 2C, left and right panels). We measured histamine levels in the hind paw tissue 1 and 6 hours after sensitization alone with either SPE-7 or ϵ 26 IgE antibodies and found no change in histamine content, indicating little to no mast cell degranulation following IgE sensitization alone (data not shown). As mast cells are capable of ‘piecemeal’ degranulation [[27] and selective mediator release [28], we assessed whether this small but detectable difference in degranulation could be associated with differential leukotriene release in SPE-7- versus ϵ 26-sensitized mice after DNP challenge, but found no differences in leukotriene B4 in the paw tissue of mice treated with either anti-DNP IgE antibodies (data not shown). Therefore the differences in pain we observe between SPE-7/DNP- and ϵ 26/DNP-treated mice are not attributable to differential neutrophil influx or rapid mast cell granule release.

3.3. SPE-7 and ϵ 26 sensitization induce equivalent cytokine responses in vivo with and without DNP challenge

It has been previously shown that SPE-7 sensitized BMCMCs secrete more IL-6 compared to ϵ 26-sensitized BMCMCs without DNP stimulation *in vitro* [8]. However, no comparisons of antigen-dependent or antigen-independent cytokine production *in vivo* by these anti-DNP IgE clones have yet been reported. Early pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α and CXCL1 are established modulators of acute inflammatory pain [29] and can all be released by mast cells [11]. Accordingly, we asked whether the increased thermal sensitivity in SPE-7-sensitized mice could be associated with increases in these cytokines following DNP challenge.

Both ϵ 26-sensitized and SPE-7-sensitized mice showed an increase in gene expression of IL-1 β one hour after DNP challenge compared to sham-sensitized controls (Figure 3A, top panels) and sham-challenged controls (data not shown). Given that SPE-7 and not ϵ 26 anti-DNP IgE was associated with an increase in pain sensitivity, we were surprised to find that upregulation of IL-1 β RNA was statistically equivalent between the clones, and only ϵ 26-sensitized mice had significantly more IL-6 transcripts than sham-sensitized controls at that time point (Figure 3A, top panels). There were no differences in IL-1 β or IL-6 protein in the hind paw tissue of mice sensitized with either clone, though both showed substantial increases over saline-sensitized or sham-challenged controls (Figure 3A bottom panels). We found no consistent differences in either transcript or protein levels of TNF- α and CXCL1 in IgE/Ag-challenged mice over saline-sensitized and sham-challenged controls (data not shown).

Given that differences in inflammatory cytokine release stimulated by these anti-DNP IgE clones have been reported in antigen-independent conditions *in vitro* [8] we also examined the transcript and protein levels of these cytokines on the day of sensitization in the absence of antigen. One hour after intraplantar injection, neither ϵ 26 nor SPE-7 IgE caused significantly more cytokine release than saline alone (Figure 3B, top panels). There was some upregulation of IL-1 β and IL-6 transcripts in all groups including the saline vehicle, reflective of the trauma of injection, which was resolved after 6 hours (data not shown). IL-1 β and IL-6 protein levels were not significantly elevated either one hour (Figure 3B bottom panels) or 6 hours (data not shown) after sensitization with either IgE clone.

Mast cell-derived IL-10 is associated with anti-inflammatory signaling [30]. Furthermore, several anti-nociceptive functions of IL-10 have recently been described including its negative regulation of voltage-gated sodium channels in rat dorsal root ganglia [31], and its role in the restoration of morphine-induced analgesia in the alleviation of pertussis toxin-provoked neuro-inflammation [32]. Therefore, we compared expression of IL-10 in ϵ 26- and SPE-7-sensitized mice, and found that one hour after DNP challenge, sensitized mice showed highly variable but not statistically significant increases in IL-10 RNA, and no increase in IL-10 protein. There were no differences between mice sensitized with ϵ 26 versus SPE-7 IgE clones (Figure 3A). IgE sensitization alone produced no significant overall increase in IL-10 RNA over sham-sensitized mice (Sal/DNP) and no increase in IL-10 protein (Figure 3B). We further analyzed mRNA and protein levels of IL-13, another known anti-nociceptive cytokine [29,33] and found no differences between SPE-7- and ϵ 26-sensitized mice (data not shown).

While cytokine differences, especially in the absence of antigen, have been a focus of interest in IgE-mediated activation of mast cells *in vitro*, we found no differences *in vivo* in either pro-inflammatory nor anti-inflammatory cytokines examined here in association with the differential PCA-pain behaviors induced by SPE-7 and ϵ 26 clones of anti-DNP IgE.

3.4. ϵ 26 and SPE-7 IgEs bind to the same or overlapping epitopes on DNP

SPE-7 and ϵ 26 were each isolated on the basis of their DNP-binding capacity [5,6] but it is not known whether these antibodies bind to the same or overlapping epitopes on DNP. As differences in epitope binding could potentially affect downstream signal transduction to

modulate pain outcomes, we compared the abilities of these anti-IgE antibodies to competitively bind DNP *in vitro*.

We performed an ELISA-based binding assay where increasing concentrations of non-biotinylated SPE-7 or non-biotinylated ϵ 26 IgE (as a control) were allowed to bind the DNP-coated plate prior to addition of a saturating concentration of biotinylated ϵ 26 IgE. Binding of biotinylated antibody was measured by enzyme-linked color change; percent colorimetric signal remaining after pre-incubation with non-biotinylated IgE clones was measured as indices of the ability of ϵ 26 and SPE-7 clones to block the binding of biotinylated ϵ 26 to plate-bound DNP. As expected, pre-incubation with non-biotinylated ϵ 26 IgE inhibited binding of the biotinylated ϵ 26 IgE in a concentration-dependent manner (Figure 4). SPE-7 IgE also inhibited biotinylated ϵ 26 IgE binding in a concentration-dependent manner, demonstrating that SPE-7 and ϵ 26 clonotypes have the same or overlapping epitope (Figure 4). Interestingly, SPE-7 IgE inhibited biotinylated ϵ 26 binding at concentrations lower than that at which non-biotinylated ϵ 26 inhibited its biotinylated counterpart. We fit an exponential decay equation to each data set and found that the decay constants (K) for the SPE-7 and ϵ 26 fitted curves were significantly different ($p = 0.003$), suggesting that while SPE-7 and ϵ 26 IgE may bind to DNP using the same or overlapping epitopes, SPE-7 IgE may do so with a greater affinity than ϵ 26.

In our analysis of cytokine profiles, leukocyte infiltration and mediator release, we found no DNP-dependent or independent differences in SPE-7 versus ϵ 26-sensitized mice that could be associated with differential mast cell-mediated inflammation caused by altered DNP binding between the two antibodies. However, this difference in binding affinity can potentially affect IgE/Ag reactions mediated by non-canonical components of the IgE network. Nerves and mast cells are frequently found in close proximity in the tissue [34] and cutaneous neurons have been shown to be a critical component of the PCA reaction in mice [35]. Primary sensory neurons in mice can express the high affinity receptor FcεR1 with activity localized on nerve fibers in the skin [36]. Therefore, it is possible that differences in pain signaling occur as a result of direct IgE/Ag activation of nerves without the mediation of mast cell-derived inflammatory signals. The only other instance of IgE/Ag-mediated pain reported in rodents implicated vascular mediators such as serotonin and histamine that can also be derived from non-mast cell sources including neurons [37,38]. The lack of correlation of anaphylaxis-provoked pain with mast cell-associated inflammation that we find here suggests that IgE antibodies may have broader antigen-dependent pathophysiological functions than previously considered.

4. Conclusion

Taken together, our findings support increased antigen-dependent hind paw thermal sensitivity in SPE-7 vs. ϵ 26-sensitized mice following DNP challenge. IgE-dependent differences in mast cell activation have previously been demonstrated *in vitro* (reviewed in [7]). Here, we show that clonal differences in anti-DNP IgEs can affect functional outcomes *in vivo*: sensitization with different clones of IgE against the same antigen can yield distinct, behavioral pain responses. We observed increased antigen-dependent hind paw thermal and mechanical pain sensitivity in SPE-7-sensitized mice, but not ϵ 26-sensitized mice, up to 6

hours after DNP challenge. Accompanying antigen-dependent tissue edema, neutrophil influx, histamine release, and induction of pro- and anti-inflammatory molecular mediators were equivalent for both clones. While there is strong evidence for antigen-independent differences in mast cell signaling and cytokine production by these IgEs *in vitro*, *in vivo* both the behavioral and molecular responses were strictly antigen dependent and possibly mediated through non-mast cell components of IgE signaling. We found that the IgE clone SPE-7 may have a slightly higher binding affinity for DNP compared to ϵ 26 though they both appear to bind using the same or overlapping epitopes. It is possible that this differential IgE/Ag binding may directly affect activation of cutaneous nerve fibers themselves or lead to differential neuropeptide-mediated signaling that produces differential thermal and mechanical sensitivities. Epidemiological [16], clinical [28] and experimental [15,39] intersections between atopy and chronic pain are an active area of current inquiry. While mast cells have been the direct focus of much of this work [12,13,34], our findings suggest that a broader consideration of non-canonical IgE signaling in pain responses will be useful. That allergic responses mediated by different IgE antibodies directed against the same antigen can provoke different pain outcomes indicate a fine-scale regulation of neuro-immune crosstalk that has yet to be fully explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographies

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Highlights

- IgE/Ag-driven cutaneous anaphylaxis induces thermal sensitivity in mouse hind paws
- SPE-7 anti-DNP IgE induces a stronger thermal pain response than ϵ 26 anti-DNP IgE
- Both antibodies produce comparable IgE/Ag-induced inflammation
- SPE-7 and ϵ 26 bind to similar or overlapping epitopes on DNP

Figure 1.

Sensitization with SPE-7 but not ε26 anti-DNP IgE antibodies provokes antigen-dependent thermal sensitivity in the hind paws of male ND4 mice. Mice sensitized with SPE-7 but not ε26 IgEs showed reduction in hind paw withdrawal latency from a 51°C hotplate after systemic DNP challenge at 24 hours post-sensitization (A) but not on the day of sensitization (B). Saline-sensitized mice with and without DNP challenge, and sham-challenged mice sensitized with either antibody showed no change in thermal sensitivity (A–B). SPE-7/DNP and ε26/DNP-challenged mice, but not sham-challenged mice showed equivalent increases in hind paw width on the day of challenge (C) while sensitization alone with either clone did not induce hind paw edema (D). IgE/Ag treatment with either clone induced hind paw extravasation of 0.05% Evans Blue dye co-injected with DNP while saline challenge did not result in dye extravasation in sensitized mice (E; left 4 panels); saline injection or IgE sensitization produced no visual changes in the hind paw. n = 15–63 mice per treatment (A); 9 per treatment (B), 9–42 per treatment (C) and 19 per treatment (D); data

pooled from up to 6 independent experiments. * and # indicate statistical significance with respect to Sal/DNP and ϵ 26/DNP, respectively. $p < 0.05$ [* , #], $p < 0.001$ [***, ###].

Figure 2.

ε26 and SPE-7 sensitization followed by DNP challenge induce equivalent neutrophil influx and histamine release in the hind paw. Tissue myeloperoxidase (A) and histamine (C) levels were measured in hind paw footpads from SPE-7 and ε26-sensitized mice challenged with either saline or DNP and saline-sensitized mice challenged with DNP at 1 and 6 hours after challenge. 4μm paraffin embedded tissue sections were stained with Toluidine Blue and stained mast cells were evaluated for extent of degranulation: 0 (intact) 1–10 (mild), 11–20 (moderate) and >20 (extensive) granules outside the cell boundary (B) at 1 and 6 hours after DNP or saline challenge in saline, SPE-7 or ε26 sensitized mice. n = 5–6 per treatment group, representative of 2 independent experiments (A, C); 3–4 per treatment group, representative of 1 experiment (B) *, # and § indicate statistical significance with respect to

Sal/DNP, ϵ 26/DNP and between IgE/Sal and IgE/DNP groups for each IgE clone, respectively. $p < 0.05$ [*], $p < 0.01$ [**], $p < 0.001$ [***, ###, §§§].

Figure 3.

Equivalent IL-1 β , IL-6, and IL-10 responses in ϵ 26 vs SPE-7 sensitized mice with and without DNP challenge, and with IgE sensitization alone. Hind paws were collected from SPE-7 or ϵ 26-sensitized mice one hour after saline or DNP challenge (A) or IgE sensitization alone (B) for quantification of relative abundance of cytokine mRNAs by semi-quantitative PCR normalized to β 2m and shown relative to no treatment controls (top, A and B) and protein content by ELISA (bottom, A and B). n = 3–9 per treatment group; representative of 2–3 independent experiments. * and § indicate statistical significance with respect to Sal/DNP and between IgE/Sal and IgE/DNP groups for each IgE clone

respectively. $p < 0.05$ [*], $p < 0.01$ [**], $p < 0.001$ [***]. Open end lines represent significance between any treatment groups, while capped lines represent significance between indicated groups.

Figure 4.

SPE-7 and $\epsilon 26$ IgE share the same or overlapping DNP binding epitope. DNP-coated plates were pre-incubated with non-biotinylated SPE-7 or $\epsilon 26$ IgE, washed, and re-incubated with biotinylated $\epsilon 26$. Binding was assessed using color change following exposure to Streptavidin-HRP and tetramethylbenzidine substrate. Blocking efficiency was determined as percentage of absorbance of biotinylated $\epsilon 26$ IgE bound over maximum binding. Using the exponential decay formula $y(t) = a * e^{Kt}$, non-linear curve fitting was applied to the absorbance data and best-fit values for decay coefficients K within the 95% confidence interval (indicated by dashed lines for each curve fit) were determined. ** indicates statistical significance between the decay coefficients. $p < 0.01$ [**].