



Pulmonary, Gastrointestinal and Urogenital Pharmacology

The role of IgE and repeated challenge in the induction of persistent increases in scratching behavior in a mouse model of allergic dermatitis

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ABSTRACT

Previously, we indicated that athymic BALB/c-nu/nu (nude) mice that had been repeatedly treated with 2,4,6-trinitrochlorobenzene (TNCB) failed to exhibit chronic scratching behavior in spite of the accumulation of dermal mast cells in the lesion. The mice also failed to produce specific IgE or potent dermatitis. In the present study, therefore, we aimed to examine the role of IgE and repeated hapten treatment in the induction of scratching behavior and dermatitis using nude mice and trinitrophenol (TNP)-specific IgE-transgenic mice. The ears of nude mice were treated with TNCB 6 times at intervals of 48 h, and TNP-specific IgE was administered to the mice intravenously before the sixth TNCB treatment. The nude mice that had been supplemented with IgE exhibited a persistent increase in scratching behavior and continuous degranulation of mast cells. Furthermore, a potent immediate ear swelling was induced, although no biphasic dermatitis pattern was observed. On the other hand, the IgE-transgenic mice failed to exhibit persistent increases in scratching behavior after a single TNCB treatment, although biphasic ear swelling was observed. These results indicate that specific IgE plays an essential role in the induction of persistent increases in scratching behavior and continuous degranulation of mast cells. Furthermore, repeated challenge with the hapten also plays an important role in persistent increases in scratching behavior through accumulation and continuous activation of mast cells.

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1. Introduction

Atopic dermatitis is defined as a chronic and relapsing eczematous dermatitis with severe itching. The itch is the most important problem and the induced scratching worsens the dermatitis. It is well known that the allergen-specific immunoglobulin E (IgE) levels in the sera of most patients are dramatically elevated (O'hameje et al., 2006). However, the critical role that the extremely high level of IgE in the patient's sera plays in the induction and development of the disease remains unknown.

The cutaneous response can be biphasically induced by a challenge with a hapten in mice that have been passively or systemically sensitized with a large amount of specific IgE (Ray et al., 1983; Katayama et al., 1990; Nagai et al., 1995). The first phase response, which peaks at 1 h after challenge, depends on mast cell degranulation and released mediators (Inagaki et al., 1999; Watanabe et al.,

1999; Dai et al., 2002). The second phase response is caused by proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). The peak response is observed at around 24 h after the challenge. The biphasic cutaneous response is induced by single hapten challenge and is completely dependent on specific IgE being given prior to the challenge, indicating that IgE contributes not only to the immediate phase cutaneous response but also to later phase responses. On the other hand, mast cell derived histamine is an important itch mediator and induces swelling and scratching behavior in ICR mice in the immediate phase (Inagaki et al., 2001). It is not clear, however, whether IgE contributes to the induction of scratching behavior in association with the later phase cutaneous response.

It is thought that a hapten induces a delayed-type cutaneous response in mice that have been sensitized with the hapten previously. The peak response usually appears 24 to 48 h after challenge. The delayed-type response is T cell-dependent; it can not be induced in nude mice (Fachet and Andó, 1977). In contrast, if the hapten challenge is repeated many times, changes in the characteristics of the induced cutaneous response occur. Repeated hapten challenges induce the expression of Th2 cytokine mRNA and protein, IgE production, and eosinophil accumulation in the lesion (Nagai et al.,

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1997a,b; Inagaki et al., 2006). An immediate cutaneous response is also observed. The cutaneous response caused by repeated hapten challenges is sometimes used as a model for atopic dermatitis.

Previously, we have reported that multiple hapten challenges administered to the ear of BALB/c mice induce biphasic ear swelling, IgE production, mast cell accumulation, continuous degranulation in the lesion, and a persistent increase in scratching behavior. In nude mice, however, although the accumulation of mast cells is induced, no potent ear swelling, IgE production, or chronic scratching behavior is observed (Yamashita et al., 2005, 2007).

In the present study, therefore, the role of IgE and repeated treatment with a hapten in the induction of scratching behavior and swelling was examined using nude mice and TNP-specific IgE-transgenic mice.

2. Materials and methods

2.1. Animals

Athymic female BALB/c-nu/nu (nude) mice and BALB/c (+/+) mice were purchased from Charles River Japan, Inc. (Yokohama, Japan) at the age of 6 weeks.

Trinitrophenyl (TNP)-specific IgE-transgenic mice (BALB/c background) (Matsuoka et al., 1999) at ages of 6–9 weeks were kindly provided by Dr. Matsuoka (The Tokyo Metropolitan Institute of Medical Science). The mice exhibit a triphasic cutaneous response upon challenge with TNP-conjugated ovalbumin (TNP-OVA) without passive sensitization (Sato et al., 2003). The mice were kept in an animal room at a temperature of 23 ± 2 °C and a humidity of $50 \pm 10\%$ under a 12 h light and dark cycle (lights on from 6:00 to 18:00). The mice were fed food (CE-2, Clea Japan, Inc.) and water *ad libitum*. The experimental protocols were approved by the Animal Care Committee of the Graduate School of Pharmaceutical Sciences, Nagoya City University, in accordance with the guidelines of the Japanese Council on Animal Care.

2.2. Application of TNCB

2,4,6-Trinitrochlorobenzene (TNCB) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). TNCB was dissolved in acetone/olive oil (4:1) at a concentration of 1 (w/v) %. Nude mice were initially sensitized by painting them with 1% TNCB (20 μ l) on each face of both ears. From 6 days later (day 0), the mice were repeatedly challenged by painting them with TNCB solution similarly every 48 h for 10 days. For the positive control, BALB/c mice were painted with TNCB similarly. For the negative control, nude mice had vehicle applied to their ears instead of TNCB solution. The thickness of the right ear lobe was measured with a dial thickness gauge (G-1A, Ozaki MFG. Co., Ltd, Tokyo, Japan) prior to and 1, 24, and 48 h after the final challenge of TNCB at day 10. Increased ear thickness was recorded as ear swelling. In one experiment, transgenic mice and non-transgenic littermates were treated with TNCB on each face of both ears once, and the changes in the ear swelling were evaluated at 1, 24, and 48 h after the application.

2.3. Injection of immunoglobulin E (IgE)

Normal serum and IgE serum were obtained from intact BALB/c and TNP-specific IgE-transgenic mice, respectively. The anti-TNP IgE concentrations in the sera of transgenic mice were 30–70 μ g/ml. The concentration of anti-TNP IgE in the IgE serum was adjusted to 15 μ g/ml, which is the average serum concentration in BALB/c mice treated with TNCB repeatedly.

Normal serum or IgE serum was intravenously injected into nude mice 1 day before the last challenge (day 9), and the effects of IgE transfer were observed.

2.4. Evaluation of scratching behavior

Scratching behavior was measured using the MicroAct system (Neuroscience, Tokyo, Japan), by which scratching behavior could be detected automatically and analyzed objectively (Inagaki et al., 2002, 2003). Briefly, a ring-type magnet (1.0 mm in inner diameter, 2.5 mm in outer diameter, 2.0 mm in height, 130–145 mT/cm²) was attached to both ankles with steel wire (No. 34, TOHO, Co. Ltd, Hiroshima, Japan). Ten to 15 h after the magnet attachment, each mouse was placed in a plastic chamber (11 cm in diameter and 18 cm in height) surrounded by a round coil, and the electric current induced in the coil by the movement of the magnets was amplified and recorded. Characteristic signals were identified as scratching behavior using the following parameters: threshold: 0.12 V, event gap: 0.02 s, minimum duration: 0.40 s, maximum frequency: 18.0 Hz, minimum frequency: 5.0 Hz. The measurement of scratching behavior for 12 h was started 48 h after the final application of TNCB. Mice were kept in the plastic chamber for 2 h for acclimation before observing the scratching behavior.

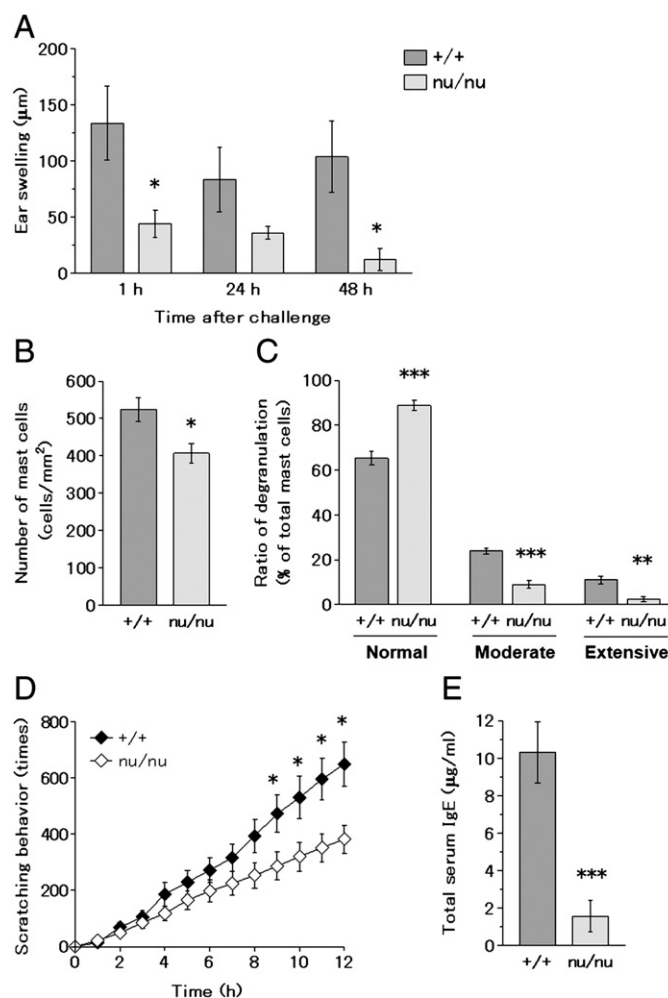


Fig. 1. A comparison of dermatitis and associated responses between BALB/c-(+/+) and BALB/c-nu/nu mice that were treated with TNCB repeatedly for 10 days at 48 h intervals. Ear swelling was measured at 1, 24, and 48 h after the final challenge (A). The number of mast cells in the dermis (B) and the proportion of degranulated mast cells (C) were counted in the ear lesion stained with acidic Toluidine Blue. Scratching behavior was observed for 12 h from 48 h after the final challenge (D). Blood samples were obtained after the measurement of scratching behavior, and total IgE levels were measured (E). Each value represents the mean $\pm$ S.E.M. of six mice (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

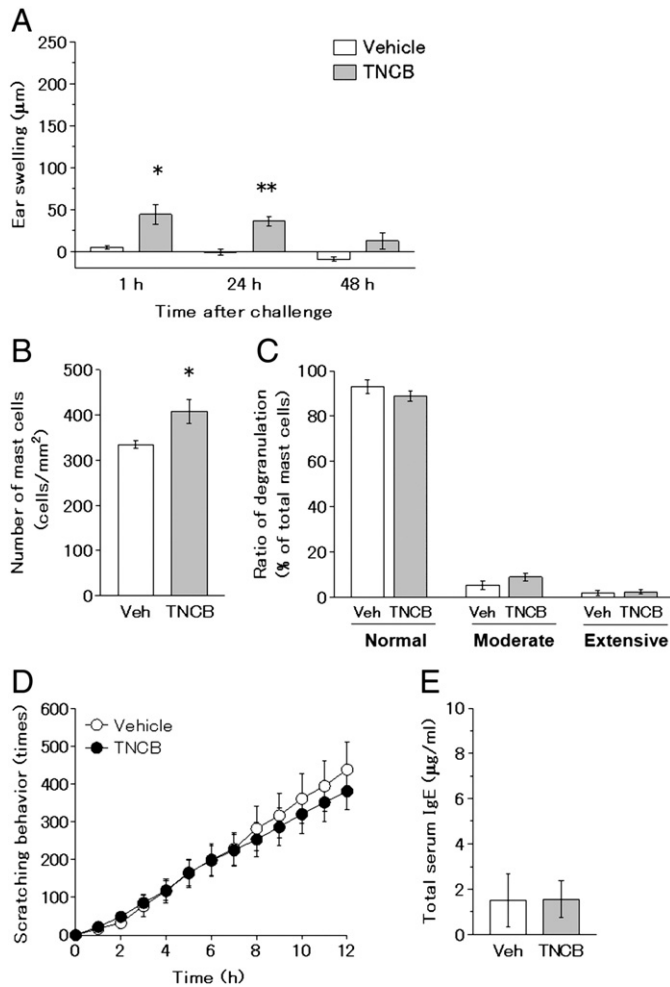


Fig. 2. A comparison of dermatitis and associated responses between BALB/c-nu/nu mice treated with TNCB and vehicle repeatedly for 10 days at 48 h intervals. The time course of ear swelling (A), the number of mast cells (B), their degree of degranulation (C), the accumulation of scratching behavior (D), and the total IgE levels (E) were measured in BALB/c-nu/nu mice treated with TNCB or vehicle. Each value represents the mean $\pm$ S.E.M. of six mice (* P < 0.05, ** P < 0.01).

2.5. Histological observation

After observing the scratching behavior, the mice were sacrificed by lethal inhalation of ether vapor. Then, the right ears of the mice were excised and fixed with 10% phosphate-buffered formalin (pH 7.2) and embedded in paraffin. Sections (3 μ m) were stained with acidic toluidine blue (pH 4.1), and the number of mast cells per square millimeter of dermis at 4 randomly chosen sites was counted. The degree of mast cell activation was evaluated as follows: extensively degranulated (extensive: >50% of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), slightly to moderately degranulated (moderate: 10–50% of the granules exhibiting fusion or discharge), and normal (Wershil et al., 1988; Yano et al., 1989).

2.6. Determination of total immunoglobulin E (IgE) levels in sera

Blood was collected from the sacrificed mice after the scratching behavior observation, and the sera were separated and stored at -20°C . The concentration of total IgE in the sera was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (Yamashita et al., 2005).

2.7. Statistics

All results are given as means $\pm$ S.E.M. Statistical analysis was conducted by the two-tailed Student's or the Aspin-Welch's t -test after the F -test to examine the homogeneity of the variance. All statistical analyses were performed by computer software, Java Applets & Servlets for Biostatistics (Programmed by H. Ono, <http://chiryo.phar.nagoya-cu.ac.jp/javastat/JavaStat-e.htm>).

3. Results

3.1. Dermatitis in nude mice treated with TNCB

The ear swelling of nude mice treated with TNCB every other day for 10 days was weaker than that of the BALB/c mice (Fig. 1A), and the ears of the nude mice treated with TNCB swelled significantly more than the ears of the vehicle-treated mice (Fig. 2A). The ear swelling of the nude mice seemed to be caused by nonspecific stimulation by the hapten because the response was weaker than that of the BALB/c mice, and the swelling at 48 h was almost negligible. The number of dermal mast cells in the ears of the BALB/c mice treated with TNCB was repeatedly significantly higher than that in the nude mice (Fig. 1B), and intense degranulation of mast cells was observed (Fig. 1C). On the other hand,

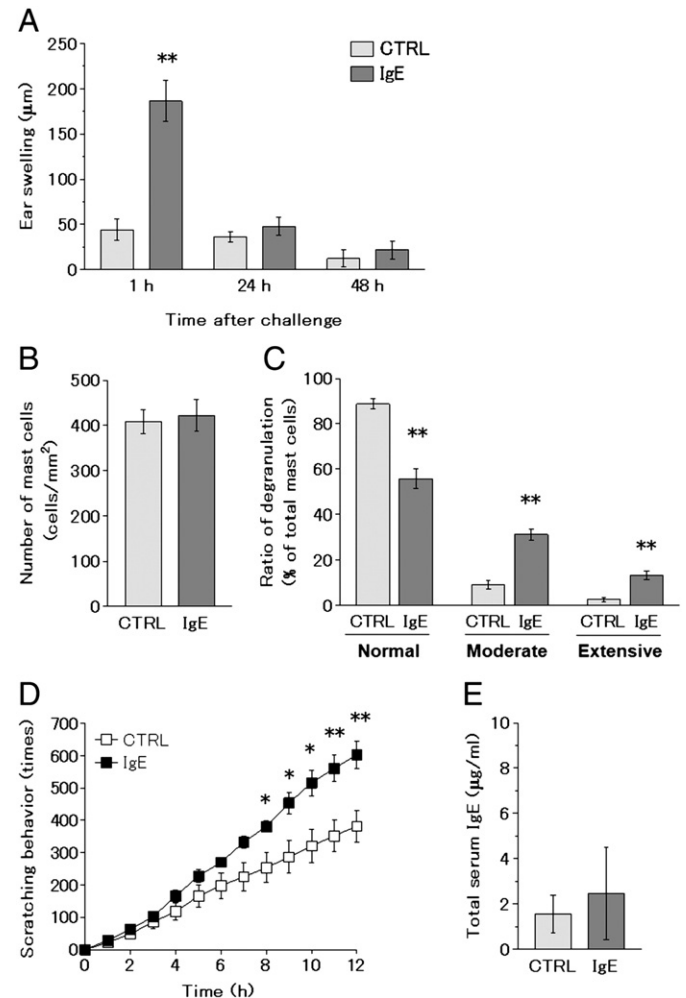


Fig. 3. The effect of TNP-specific IgE injection into BALB/c-nu/nu mice. BALB/c-nu/nu mice treated with TNCB were injected with (IgE) or without (CTRL) TNP-specific IgE. The time course of ear swelling (A), the number of mast cells (B), their degree of degranulation (C), the accumulation of scratching behavior (D), and the total IgE levels (E) were measured in BALB/c-nu/nu mice treated with TNCB with or without an IgE injection. Each value represents the mean $\pm$ S.E.M. of six mice (* P < 0.05, ** P < 0.01).

although the number of dermal mast cells in the ears of the nude mice treated with TNCB was significantly increased compared to that in the vehicle-treated mice (Fig. 2B), there was no difference between the vehicle-treated and TNCB-treated mice with regard to the levels of mast cell degranulation (Fig. 2C) and total serum IgE (Fig. 2E). Furthermore, persistent scratching was not induced in the nude mice (Fig. 2D), whereas it was observed in the BALB/c mice (Fig. 1D). Elevated serum IgE levels were also observed in the BALB/c mice (Fig. 1E).

3.2. The effects of IgE transfer into nude mice

IgE serum from TNP-specific transgenic mice was transferred into nude mice, and dermatitis caused by repeated application of TNCB was observed. The ear swelling in the IgE-injected nude mice was significant at 1 h compared to that in the control mice, but no swelling was apparent at 24 or 48 h (Fig. 3A). Although the number of mast cells in the ears of the IgE-injected nude mice was comparable to that in the control mice, the proportion of degranulated mast cells increased, and concomitantly that of the intact mast cells also decreased (Fig. 3B, C). Furthermore, a persistent increase in scratching behavior was observed in the nude mice that had been injected with IgE (Fig. 3D). There were no differences between the IgE-injected and

control mice with regard to the serum collected after the measurement of scratching behavior (Fig. 3E).

3.3. Dermatitis in transgenic mice after a single application of TNCB

The effect of a single application of TNCB was observed in IgE-transgenic mice. In the wild type mice, a single TNCB application caused slight swelling at 1, 24, and 48 h (Fig. 4A). In the transgenic mice, potent swelling was observed at 1 h after the application and the swelling at 48 h was slightly more potent than that in the wild type mice (Fig. 4A). However, no increase in mast cell number or degranulation rate was evoked, nor was a persistent increase in scratching behavior observed (Fig. 4B–D). The total IgE levels in the wild type mice after a single application of TNCB were lower than those in the transgenic mice (Fig. 4E).

4. Discussion

Previously, we demonstrated that repeated application of TNCB to BALB/c mice causes a persistent increase in scratching behavior that is associated with the accumulation and continuous degranulation of mast cells, and elevated serum IgE levels. Athymic nude mice, however, do not exhibit a persistent increase in scratching behavior even after repeated treatment with TNCB in spite of the accumulation of mast cells in the lesion. These mice also fail to respond to the hapten during the production of IgE (Yamashita et al., 2005).

In the present study, therefore, we attempted to find out whether nude mice exhibit a persistent increase in scratching behavior when specific IgE is administered to them. At first, we confirmed our previous observation that nude mice fail to develop ear swelling and IgE production after repeated TNCB challenges, demonstrating that these responses are T cell-dependent (Fig. 2A, E). Furthermore, although a slight increase in mast cell number was induced in the lesions of the nude mice, no continuous degranulation of mast cells was observed (Fig. 2B, C), indicating that mast cell activation is dependent on specific IgE. No persistent increase in scratching behavior was observed in the nude mice even after repeated TNCB challenges (Fig. 2D). Therefore, continuous mast cell activation seems to be essential for the persistent increase in scratching behavior that is observed in BALB/c mice (Fig. 1C, D).

So, to confirm the role of IgE in the induction of ear swelling and persistent increases in scratching behavior, the effects of IgE transfer into nude mice treated with TNCB were repeatedly examined. TNP-specific IgE was injected intravenously into nude mice 24 h after the fifth challenge with TNCB. Upon the final challenge, the IgE-injected mice exhibited a potent ear swelling at 1 h (Fig. 3A). Furthermore, continuous mast cell degranulation and increased persistent scratching were also observed (Fig. 3C, D). These results clearly demonstrate that IgE is an important factor in the causation of ear swelling and scratching behavior in this dermatitis model. The serum IgE levels in the IgE-injected mice after the observation of scratching behavior were comparable to those in the control mice. Therefore, IgE sensitization seems to be essential. The low levels of serum IgE in the IgE-injected mice might have resulted from its rapid adsorption by its high affinity receptors and its short half-life. Because these responses depend on IgE, we consider that not only T cells but also B cells, which produce IgE, are important cells in these reactions. Repeated treatment with TNCB shifts from Th1 dominant response to Th2 (Kitagaki et al., 1995, 1997). Interleukin-4 (IL-4) of Th2 cytokine is induced in the inflammatory skin. And IL-4 produced by activated T cells effects IgE synthesis in B cells (Bacharier et al., 1998). So, the serum IgE level in the TNCB-treated nude mice did not increase (Fig. 2E).

Next, the role of repeated hapten challenges in ear swelling and scratching behavior was examined in IgE-transgenic mice. The transgenic mice with high serum levels of TNP-specific IgE were stimulated with TNCB once. Although the single stimulation with

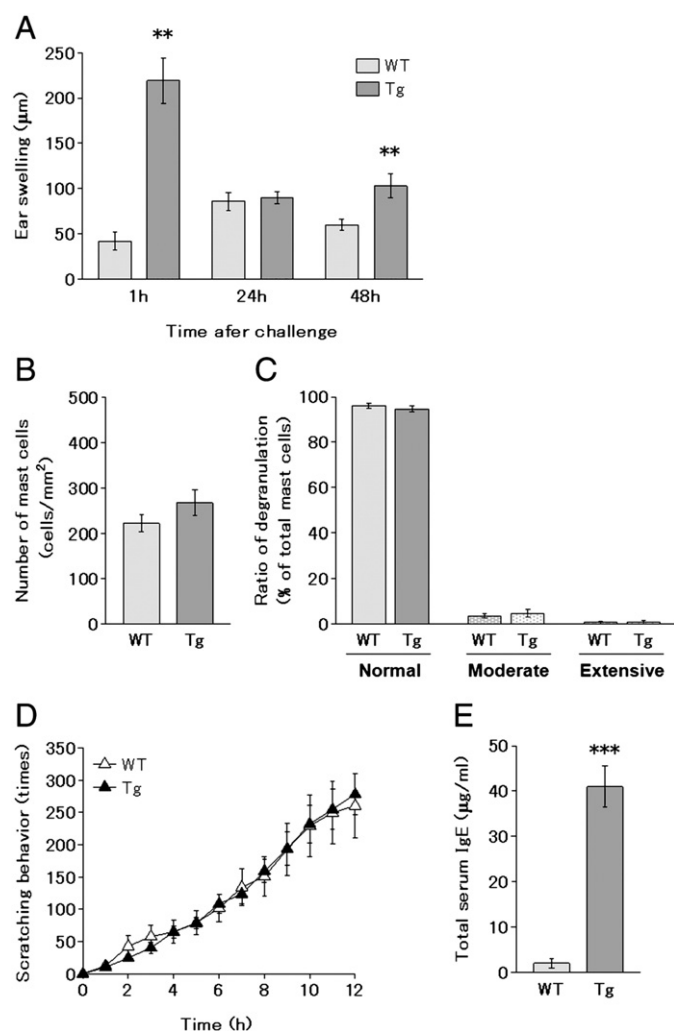


Fig. 4. A comparison of dermatitis and associated responses between the transgenic mice (Tg) that constantly expressed high-doses of TNP-specific IgE and their non-transgenic littermates (WT) that were treated with TNCB once. The time course of ear swelling (A), the number of mast cells (B), their degree of degranulation (C), the accumulation of scratching behavior (D), and the total IgE levels (E) were measured in transgenic mice or their littermates. Each value represents the mean $\pm$ S.E.M. of nine mice (** $P < 0.01$, *** $P < 0.001$).

TNCB caused potent ear swelling at 1 h in the transgenic mice (Fig. 4A), neither continuous mast cell degranulation nor a persistent increase in scratching behavior could be induced (Fig. 4C, D).

These results indicate that a single challenge with the hapten is not enough to induce continuous mast cell degranulation and scratching behavior even in the presence of sufficient amounts of specific IgE. Therefore, repeated TNCB challenges as well as IgE sensitization seem to be important. Repeated TNCB-treatment induces inflammation in the ear, and repeated proinflammatory stimuli and prolonged inflammation result in an accumulation of mast cells in the skin lesion. Many endogenous factors appear to be involved in mast cell accumulation. Repeated mast cell stimulation may also be important for the accumulation. Well known mast cell growth factors such as stem cell factor (SCF), interleukin-3 (IL-3), and interleukin-4 (IL-4) may also be involved (Tsuji et al., 1990, 1995; Takagi et al., 1992). The endogenous factors responsible for mast cell accumulation may also be responsible for the continuous activation of mast cells. Therefore, repeated hapten stimulation may be important not only for mast cell accumulation but also for continuous degranulation of mast cells upon hapten stimulation. As a result, continuous degranulation of mast cells induces persistent increases in scratching behavior.

In conclusion, repeated TNCB stimulation causes dermatitis with a persistent increase in scratching behavior in BALB/c mice. Although repeated TNCB challenge induces IgE production, it also induces mast cell accumulation and continuous mast cell degranulation even in the absence of IgE. However, no persistent increase in scratching behavior could be induced in the absence of IgE. IgE is essential and sufficient to induce the immediate cutaneous response. IgE is also essential but not sufficient to induce a persistent increase in scratching behavior. These results clearly demonstrate that repeated hapten challenges and the resultant elevation of serum IgE levels are essential for the induction of persistent increases in scratching behavior.

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