

Chronic inflammation of the skin can be induced in IgE transgenic mice by means of a single challenge of multivalent antigen

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Background: It is now widely accepted that IgE mediates immediate-type allergic response. However, the pathologic role of IgE is controversial in the chronic allergic inflammation observed in atopic diseases, such as asthma and atopic dermatitis.

Objective: We investigated the role of IgE in cutaneous allergic reactions by using 2 newly developed lines of antigen-specific IgE transgenic mice.

Methods: IgE transgenic mice were administered subcutaneously with corresponding antigens, and the subsequent ear swelling was measured.

Results: A single subcutaneous administration of TNP-conjugated ovalbumin (OVA) into the ears of nonimmunized mice carrying the TNP-specific IgE transgene elicited immediate-phase and late-phase ear swelling as expected, which peaked at 20 minutes and 8 hours later, respectively. Interestingly, however, 2 to 3 days after the antigen challenge, more intense ear swelling appeared. Its magnitude and duration were dependent on the valency of TNP in OVA, as well as the dose of TNP-OVA, and it lasted over 1 month when 100 µg of OVA conjugated with 11 molecules of TNP was given. Interestingly, administration of OVA to OVA-specific IgE transgenic mice elicited immediate-phase and late-phase ear swelling but not third-phase ear swelling. Massive infiltration of inflammatory cells was observed in the third-phase ear swelling of TNP-specific IgE transgenic mice. Cyclosporine A almost completely inhibited the third-phase ear swelling and cellular infiltration, whereas an antihistamine, cyproheptadine, did not show any significant effect on the third-phase reaction.

Conclusion: These results indicate that IgE can trigger not only immediate-type hypersensitivity but also chronic allergic inflammation. Our findings highlight a novel immunopathologic role of IgE in chronic atopic disorders. (*J Allergy Clin Immunol* 2003;111:143-8.)

Key words: IgE, transgenic, atopic dermatitis, chronic inflammation, skin, mouse, multivalent antigen, FcεRI

IgE was identified in 1967 as the antibody responsible for early allergen-induced wheal-and-flare reactions in the skin.¹ It is now widely accepted that IgE mediates immediate hypersensitivity, so-called type I allergy.² The event of antigen-IgE-mediated mast cell degranulation is the first step in allergic reaction and results in prompt release of a series of chemical mediators, such as histamine and serotonin, as well as a variety of enzymes that play important roles in initiating allergic-type and anaphylactic-type responses. On the contrary, the pathologic role of IgE is controversial in chronic allergic inflammation observed in atopic diseases, such as asthma and atopic dermatitis. It has been generally considered that chronic allergic inflammation is mainly mediated by T cells rather than by IgE and mast cells. Recently, humanized anti-IgE mAb (omalizumab) was developed and clinically applied to patients with allergic asthma.³ Clinical studies have shown that this mAb attenuates both immediate and delayed inflammatory airway responses, suggesting that IgE might mediate allergic reactions beyond type I allergy.

To investigate the possible immunopathologic roles of IgE in vivo, we have established 2 lines of antigen-specific IgE transgenic mice, one specific for hapten 2,4,6-trinitrophenol (TNP) and the other specific for the protein allergen ovalbumin (OVA). They constantly produce high titers of antigen-specific IgE, and their mast cells are heavily loaded with IgE. Without any prior sensitization, immediate allergic reactions, such as systemic anaphylaxis, can be elicited when transgenic mice are challenged with the corresponding antigen.⁴ In the present study, by using transgenic mice, we show that IgE has a critical role in the development of not only immediate allergic reactions but also very delayed inflammatory reactions when multivalent antigens are involved. These findings present a novel pathologic role of IgE in chronic allergic inflammation and provide a new insight into the cause of atopic diseases.

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Abbreviations used

OVA: Ovalbumin
TNP: Trinitrophenol

METHODS**IgE transgenic mice**

IgE transgenic mice, which express H and L chains of TNP-specific IgE, were established as previously described.⁴ OVA-specific IgE transgenic mice were established on the basis of the method we established for TNP-specific IgE transgenic mice as follows: cDNA fragments corresponding to variable regions of the H and L chains of anti-OVA IgE were cloned by means of RT-PCR from an anti-OVA IgG-producing hybridoma, OVA 01-4. By using these cDNA fragments as probes, a 1.8-kbp *NcoI-XbaI* fragment carrying a leader exon and a rearranged VH_{DHJH} exon and a 2.1-kbp *PvuII-PstI* fragment carrying a leader exon and a rearranged VκJκ exon were isolated from the OVA 01-4 genomic DNA library. The anti-OVA IgE H and L chain constructs were prepared as previously described⁴ and microinjected into fertilized eggs of BALB/cCrSlc mice (Japan SLC, Hamamatsu, Japan), followed by transfer of viable eggs into the oviducts of pseudopregnant ICR mice (Japan SLC). Male IgE transgenic mice were mated with female BALB/c mice, and progenies were screened on the basis of serum IgE (>1 μg/mL). The animal experiments were conducted in accordance with the guidelines for animal use and experimentation, as set out by our institutions.

Antigens

OVA conjugated with 11, 8, or 5 molecules of TNP (TNP₁₁-OVA, TNP₈-OVA, or TNP₅-OVA, respectively) was purchased from Biosearch Technologies (Novato, Calif). OVA conjugated with other numbers of TNP molecules was prepared as previously described.⁵ OVA (Sigma, St Louis, Mo) was incubated overnight with various concentrations of 2,4,6-trinitrobenzenesulfonic acid (Nacalai Tesque, Kyoto, Japan) and then fractionated with a Sephadex G25 column (PD-10; Amersham Biosciences, Uppsala, Sweden). OVA conjugated with 2 or 10 molecules of TNP per protein (TNP₂-OVA or TNP₁₀-OVA, respectively) was used for the experiments. BSA was purchased from Sigma.

Antigen-induced ear swelling

The right ears of transgenic mice and nontransgenic littermates were subcutaneously administered 10 μL of specific antigen (TNP-OVA or OVA) solution, and their left ears were administered an equal volume of control protein (OVA or BSA) solution by using a microsyringe after achievement of light anesthesia with ether. The dose of antigen was 1 μg per site unless otherwise stated. Ear thickness was measured with a dial thickness gauge (G-1A; Ozaki, Tokyo, Japan) in units of 10⁻² mm. Four mice were used in each group. Data are expressed as the mean ± SEM of 4 mice in a representative experiment from 10 repeated experiments. Statistical analysis was performed in each experiment.

Histologic examination

Mice were killed by means of carbon dioxide gas, and ear specimens were fixed in 10% formalin. The fixed ear specimens were embedded in paraffin, and sliced sections were stained with hematoxylin and eosin. Individual cell counts were performed by using 4 high-power fields under a microscope and expressed as the mean ± SEM. The sliced sections were also stained with toluidine blue for counting the mast cells.

Evaluation of the effects of an antihistamine and an immunosuppressant

TNP-specific IgE transgenic mice were administered with TNP₁₁-OVA, as described above. Cyproheptadine hydrochloride (Sigma) was dissolved in 20% 2-hydroxypropyl-β-cyclodextrin (Nihon Shokuhin Kako, Tokyo, Japan) and orally administered (10 mg·kg⁻¹·d⁻¹) 6 times, namely 1 day before, 1 hour before, and 1, 2, 3, and 4 days after antigen challenge. Cyclosporine A solution (Sandimmun; Novartis Pharma, Tokyo, Japan) was diluted in saline solution and orally administered (30 mg·kg⁻¹·d⁻¹) at the same time points. Inhibitory effects of these drugs at the indicated time points were calculated as follows:

$$\text{Inhibitory effect (\%)} = (1 - [A - B]/[C - D]) \times 100,$$

where A is the mean ear thickness of the right ear in treated mice, B is the mean ear thickness of the left ear in treated mice, C is the mean ear thickness of the right ear in control mice, and D is the mean ear thickness of the left ear in control mice.

Statistics

Significance of difference was determined by using the *t* test with the computer software SAS (version 8.0; SAS Institute Inc, Cary, NC).

RESULTS**Antigen-specific and IgE-dependent triphase ear swelling in IgE transgenic mice**

Two lines of antigen-specific IgE transgenic mice were administered subcutaneously with corresponding or control antigens to investigate the role of IgE in cutaneous allergic reactions, and the subsequent ear swelling was measured. The administration of TNP₁₁-OVA (1 μg) to ears of TNP-specific IgE transgenic mice elicited immediate-phase ear swelling, which peaked 20 minutes after the antigen challenge, followed by weak late-phase ear swelling, which peaked 8 hours later (Fig 1, A). After these 2 peaks of ear swelling, a very strong third-phase ear swelling was observed from 2 or 3 days after the antigen administration, which peaked on day 4 (Fig 1, A). No significant ear swelling was detected in the left ear, in which OVA in place of TNP₁₁-OVA was injected. Furthermore, neither TNP₁₁-OVA nor OVA elicited significant ear swelling in nontransgenic control mice (Fig 1, B). These results indicated that the third-phase ear swelling was both antigen specific and IgE dependent.

In contrast, the administration of OVA (1 μg) to OVA-specific IgE transgenic mice elicited immediate-phase and late-phase ear swelling but not third-phase ear swelling (Fig 1, C), even though levels of antigen-specific IgE in their sera were comparable with those of TNP-specific IgE transgenic mice (20–40 μg/mL). This was also the case when 1 μg of TNP₁₁-OVA in place of OVA was given to OVA-specific IgE transgenic mice; however, this dose elicited third-phase ear swelling in the TNP-specific IgE transgenic mice. Even higher doses of up to 100 μg of OVA did not induce third-phase ear swelling in the case of OVA-specific IgE transgenic mice (data not shown). Because monoclonal anti-OVA IgE recognizes an epitope on OVA, administered OVA is not capable of cross-linking anti-OVA IgE molecules on mast cells in theory. However,

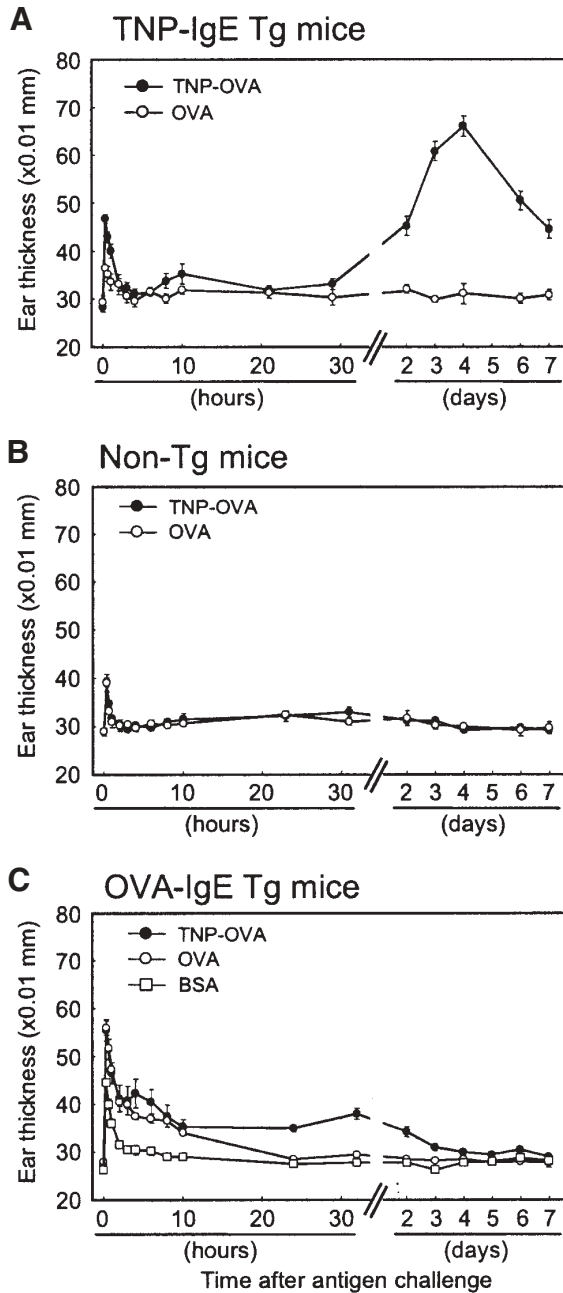


FIG 1. Antigen-specific and IgE-dependent triphase ear swelling in TNP-specific IgE transgenic mice. **A**, Ear thickness of TNP-specific IgE transgenic mice was measured at the indicated time points after subcutaneous administration of 1 μ g of TNP₁₁-OVA or OVA. **B**, Ear thickness of nontransgenic normal mice after administration of 1 μ g of TNP₁₁-OVA or OVA. **C**, Ear thickness of OVA-specific IgE transgenic mice after administration of 1 μ g of TNP₁₁-OVA, OVA, or BSA. Data are expressed as the mean $\pm$ SEM of 4 mice and are representative of 10 repeated experiments.

intravenous injection of OVA induced typical systemic anaphylaxis, with a drastic drop in body temperature in OVA-specific IgE transgenic mice (data not shown). Therefore it appears that aggregated OVA cross-linked IgE on mast cells and induced immediate allergic reaction in

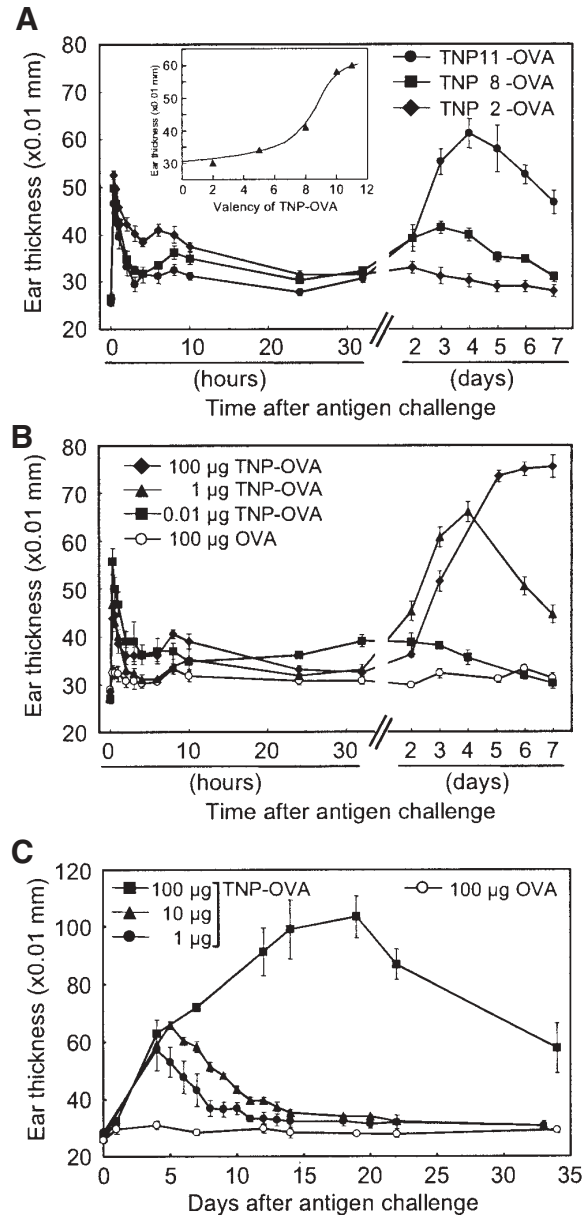


FIG 2. Third-phase ear swelling is dependent on the valence and dose of antigen. **A**, Ear thickness of TNP-specific IgE transgenic mice after administration of 1 μ g of TNP-OVA of different valencies, as indicated. In the *inset* ear thickness on day 4 is plotted against the valency of TNP-OVA. **B** and **C**, Ear thickness of the transgenic mice after administration of the indicated doses of TNP₁₁-OVA or OVA. Data are expressed as the mean $\pm$ SEM of 4 mice and are representative of 10 repeated experiments.

vivo. Thus the presence or absence of third-phase ear swelling in antigen-specific IgE transgenic mice might be attributable to the valence of the antigen: high valence in TNP₁₁-OVA versus low valence in OVA.

Third-phase ear swelling is dependent on the valence and dose of the antigen

To clarify the effect of the valence of the antigen on

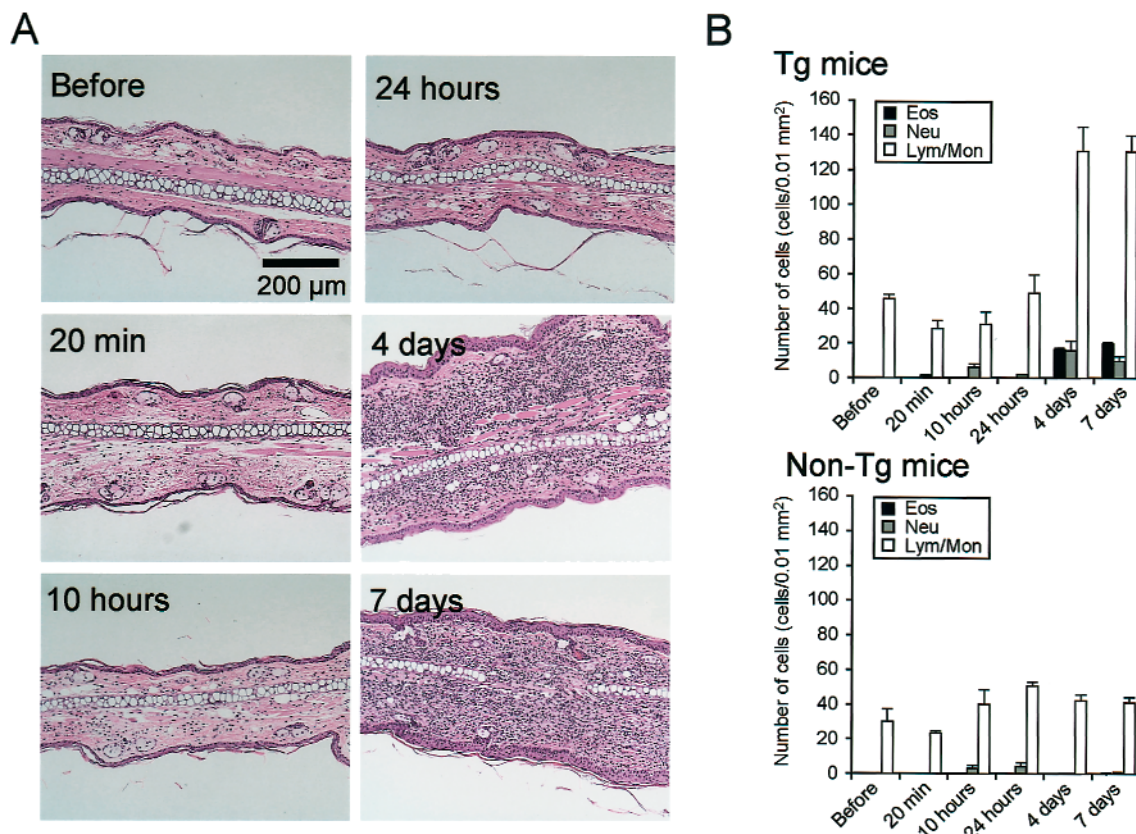


FIG 3. Massive cellular infiltration in swollen ear skin in the third-phase reaction. **A**, Ear specimens were prepared from TNP-specific IgE transgenic mice and stained with hematoxylin and eosin at the indicated time points after the administration of 1 μ g of TNP₁₁-OVA. **B**, Differential cell counts were performed on the ear specimens of the transgenic and nontransgenic mice. Data are expressed as the mean $\pm$ SEM of 4 high-power fields. Eos, Eosinophil; Neu, neutrophil; Lym, lymphocyte; Mon, monocyte.

the incidence of third-phase ear swelling, we compared OVA molecules conjugated with different numbers of TNP in their ability to induce the third-phase ear swelling (Fig 2, A). TNP₁₀-OVA elicited third-phase ear swelling in TNP-specific IgE transgenic mice to the same degree as TNP₁₁-OVA, whereas TNP₈-OVA elicited much milder ear swelling. TNP₂-OVA and TNP₅-OVA elicited little or no third-phase ear swelling, despite eliciting immediate-phase and late-phase ear swelling. Thus the third-phase ear swelling apparently depended on the valency of TNP-OVA. The magnitude and duration of the third-phase ear swelling elicited by TNP₁₁-OVA in the TNP-specific IgE transgenic mice were found to be dependent on the antigen dose (Fig 2, B and C). When 1 and 10 μ g of TNP₁₁-OVA was given, ear thickness reached a maximum at 0.57 and 0.66 mm on days 4 and 5 and subsided by days 12 and 14, respectively (Fig 2, C). When 100 μ g of TNP₁₁-OVA was administered, ear thickness reached 1.04 mm on day 19, and the ear swelling lasted for longer than 1 month. In contrast, 0.01 μ g of TNP₁₁-OVA elicited only very mild third-phase ear swelling, even though it was sufficient to induce immediate-phase ear swelling (Fig 2, B).

Massive cellular infiltration in swollen ear skin in the third-phase reaction

Ear specimens were prepared at the time points indicated in Fig 3, A, after administration of 1 μ g of TNP₁₁-OVA to investigate the triphase cutaneous reaction histologically. Although ear swelling was observed at 20 minutes and 8 to 10 hours after antigen challenge, no significant infiltration of inflammatory cells was observed at these time points or at 24 hours after antigen challenge. Four days after antigen challenge, namely when ear swelling reached the maximum level, strong cellular infiltration was observed in the dermis. The infiltrated cells were mostly mononuclear cells (lymphocytes and monocytes), and eosinophils and neutrophils were also significantly detected (Fig 3, B). The number of infiltrated cells markedly increased on day 4 in accordance with the ear swelling and continued to day 7 after the antigen challenge. Thickening of the epidermis was also observed at the same time. The number of mast cells in the dermis did not change significantly throughout the triphase reaction (data not shown). No significant histologic change was detected in the ears of nontransgenic control mice, except

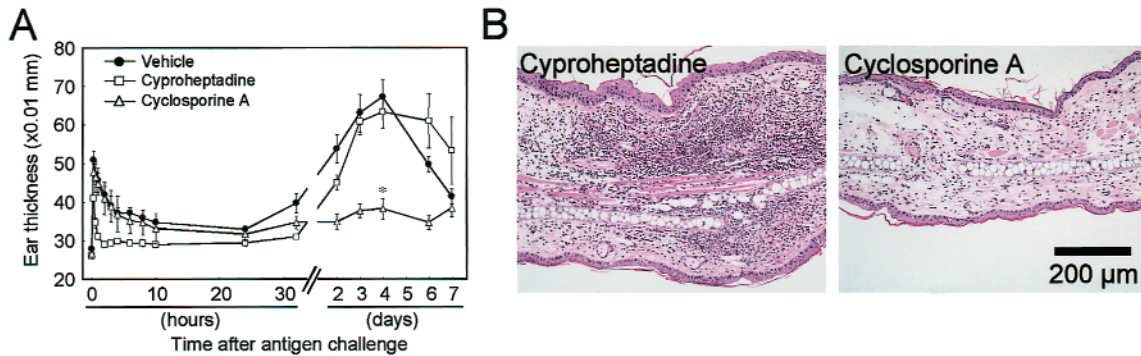


FIG 4. An immunosuppressant but not an antihistamine inhibited third-phase ear swelling. **A**, Ear thickness of TNP-specific IgE transgenic mice after administration of 1 μ g of TNP₁₁-OVA. Cyproheptadine (10 mg·kg⁻¹·d⁻¹), cyclosporine A (30 mg·kg⁻¹·d⁻¹), or vehicle was orally administered for 5 days from 1 day before to 4 days after the antigen challenge. Data are expressed as the mean $\pm$ SEM of 4 mice and are representative of 10 repeated experiments. * P < .05 versus vehicle. **B**, Cross-sections of ears on day 4 after the antigen challenge are shown.

nonspecific swelling 20 minutes after subcutaneous administration of antigen (data not shown).

An immunosuppressant but not an antihistamine inhibited third-phase ear swelling

The effect of an antihistamine was analyzed to investigate whether the third-phase ear swelling involved immediate mediators originating in mast cells or basophils. Because both histamine and serotonin have been shown to mediate the immediate allergic response in mice,⁶ cyproheptadine, which has antihistaminic, as well as antiserotonin, activity, was chosen as the antihistamine. In parallel the effect of an immunosuppressant, cyclosporine A, was also examined. TNP-specific IgE transgenic mice were treated orally with cyproheptadine or cyclosporine A for 5 days starting 1 day before administration of 1 μ g of TNP₁₁-OVA. Cyproheptadine strongly suppressed the immediate-phase ear swelling (inhibitory rate, 89% at 20 minutes after antigen challenge) but did not show any significant effect on the third-phase ear swelling (6% on day 4; Fig 4, A). In contrast, cyclosporine A significantly suppressed the third-phase ear swelling (80% inhibition, P < .05 vs control value) and suppressed the immediate-phase ear swelling to a lesser extent (33% inhibition). Histologic examination revealed that cyclosporine A drastically reduced infiltration of inflammatory cells in the third-phase ear swelling (Fig 4, B).

DISCUSSION

The present study clearly demonstrated the possible role of IgE in triggering chronic allergic inflammation in skin. A single challenge of the multivalent antigen TNP₁₁-OVA induced a very strong, long-lasting, third-phase ear swelling after the immediate-phase and late-phase ear swelling in an antigen-specific and IgE-dependent manner in IgE transgenic mice. The occurrence of the third-phase ear swelling was dependent on both the valency and dose of the antigen and was not simply correlated with the potency of the antigen to induce an

immediate-phase allergic reaction. Neither TNP-OVA with low valence nor low-dose TNP₁₁-OVA could induce the third-phase ear swelling, even though they were sufficient to induce the immediate-phase ear swelling. Therefore intense aggregation of IgE and Fc ϵ RI on mast cells with multivalent antigens appears to be necessary to trigger third-phase ear swelling.

Tahara et al⁷ reported that epicutaneous 2,4-dinitrofluorobenzene challenge to mice passively sensitized with dinitrophenol-specific IgE induced very late-phase ear swelling that peaked on day 8 after the antigen challenge. However, the magnitude of ear swelling was 5 to 10 times less in their study than in our system. Furthermore, in their study even nonsensitized mice showed ear swelling, more than half as much as observed in the sensitized mice. Therefore it is not clear whether the third-phase ear swelling response in their study is the same in nature as that we observed in the TNP-specific IgE transgenic mice in our study. In our system, using TNP-specific IgE transgenic mice, epicutaneous challenge with picryl chloride elicited immediate-phase and late-phase ear swelling but not third-phase ear swelling, most likely because of the low valence or low dose of haptenated proteins in the skin.

The third-phase ear swelling observed in the IgE transgenic mice was almost completely suppressed by cyclosporine A but not by cyproheptadine. It has been reported that cyclosporine A inhibits Fc ϵ RI aggregation-dependent expression of chemokine and cytokine but not the exocytosis of chemical mediators, such as histamine, in mast cells.^{8,9} Therefore it is possible that some chemokines and cytokines released from mast cells are involved in the recruitment of inflammatory cells observed in third-phase ear swelling. Torigoe and Nakanishi¹⁰ reported that the intensity of calcium signal transduction transmitted from Fc ϵ RI cross-linking on mast cells depends on the hapten density of the antigen molecules. Strong and stable aggregation of IgE-Fc ϵ RI on mast cells with multivalent antigens might lead to the production of much larger amounts of chemokines and

cytokines or different kinds of mediators compared with the milder aggregation of IgE-FcεRI with low-valent antigens. Cyclosporine A might have suppressed the third-phase ear swelling by inhibiting the production of those mediators. Alternatively, but not mutually exclusively, cyclosporine A might have exhibited the anti-inflammatory effect through inhibition of calcineurin-dependent T-cell activation. In this regard it should be noted that the third-phase ear swelling observed in the IgE transgenic mice is not a typical delayed-type hypersensitivity reaction because the mice had never been immunized with the antigen before the antigen challenge. Before antigen challenge, submental lymph node cells isolated from TNP-specific IgE transgenic mice did not show any proliferative response to TNP-OVA in vitro, as in the case of those isolated from nontransgenic mice (unpublished observation, December 11, 2000). After antigen challenge, a proliferative response was observed, which peaked on day 4 and subsided by day 7. However, this proliferative response is not specific for transgenic mice because a comparable response was observed in lymph node cells from nontransgenic mice challenged with the same antigen. Furthermore, there was no significant difference between transgenic and nontransgenic mice in the proliferative response of their lymph node cells to the T-cell mitogen concanavalin A (unpublished observation, May 17, 2002). Thus T cells in transgenic mice were not hyperreactive and responded normally to mitogen and antigens as far as we examined. Of course, our results do not exclude the important role of T cells in the third-phase ear swelling. We would like to emphasize that the third-phase response could be elicited in transgenic mice but not in nontransgenic mice. Moreover, when OVA-TCR transgenic mice were challenged with a subcutaneous injection of TNP₁₁-OVA into the ears, the third-phase ear swelling was not observed (unpublished observation, July 31, 2000). Therefore we assume that the third-phase response is not solely dependent on T cells and that mast cells possibly contribute to the third-phase response. The subject of which type of cells is involved in the third-phase reaction is under investigation by crossing the transgenic mice with other mutant mice, such as T cell- or mast cell-deficient mice.

The histologic features observed in the third-phase ear swelling appear similar to those of the lichenoid eczema of atopic dermatitis, as regards infiltration of mononuclear cells and granulocytes and thickening of the epidermis. The inhibitory effect of cyclosporine A to third-phase ear swelling corresponds with the clinical efficacy of cyclosporine A in atopic dermatitis.¹¹ Therefore the IgE-dependent chronic inflammatory reaction observed in the IgE transgenic mice possibly contributes to the pathology of atopic dermatitis. It was reported that some natural allergens have multiple epitopes recognized by IgE. α-Lactalbumin and β-lactoglobulin have 4 epitopes each,¹² and a grass pollen allergen includes at least 9 epitopes, several of which were simultaneously recognized by the serum of individual patients.¹³ Therefore natural allergens could work as multivalent antigens in allergic

patients. Furthermore, it is not unusual that the clinical allergic patch test reaction persists over 14 days if the reaction is monitored for a long period.¹⁴ The prevalence rate of the long-lasting allergic patch test reaction in atopic patients is reportedly higher than that in nonatopic patients. Thus it is possible that multivalent antigens elicit chronic allergic reaction in cooperation with high IgE, especially in atopic patients.

In summary, the present study highlights a novel immunopathologic role of IgE in chronic allergic inflammation and provides a new insight into the cause of atopic diseases, such as atopic dermatitis. Further analysis of the triphase ear thickening reaction in IgE transgenic mice could lead to the discovery of novel therapeutic targets for treating chronic allergic inflammation.

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