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Roles of TNF-α and IgE in the late phase of contact hypersensitivity induced by trimellitic anhydride

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Abbreviations: A/O, acetone: olive oil solution; CHS, contact hypersensitivity; ESR, ear swelling responses; PMNLs, polymorphonuclear leukocytes; TMA, trimellitic anhydride

Abstract

Trimellitic anhydride (TMA) is widely used industrially to make epoxy and alkyd resins, plasticizers and surfactants. The purpose of this study was to investigate whether contact hypersensitivity (CHS) is induced by repeated TMA challenge and the role of TNF-α and IgE in the TMA-induced CHS. The repetition of the challenge enlarged the extent of an early and a late phase of CHS in TNF-$\alpha^{+/+}$ (B6129SF2/J) and Balb/c mice. In the late phase of TMA-induced CHS, the peak of ear swelling responses by single challenge showed at 24 h after challenge, but the peak was observed at 8 h after repeated challenge. In the TNF-α knockout TNF-$\alpha^{-/-}$ (B6;129S-Tnf^{tm1Gk1}) mice, the repetition of the TMA challenges enlarged the extent of the late phase of CHS, but less than those in TNF-$\alpha^{+/+}$ mice. Injection of anti-TNF-α antibody into the peritoneal cavity of Balb/c mice significantly decreased the extent of the late phase of CHS. Subcutaneous injection of anti-IgE antibody into Balb/c mice also decreased the extent of the late phase of CHS in dose-dependent manner. Histologically, infiltration of polymorphonuclear leukocytes and eosinophils was more pronounced in repeatedly TMA-challenged TNF-$\alpha^{+/+}$ and Balb/c mice than in the TNF-$\alpha^{-/-}$ mice and anti-TNF-α or anti-IgE antibodies treated Balb/c mice. These results indicate that mice sensitized by TMA could possibly offer a useful model to study the mechanism of CHS, and TNF-α and IgE may act as potential modulators in the late phase of TMA-induced CHS. Neutralization of TNF-α and IgE by anti-TNF-α or anti-IgE antibodies may provide therapeutic tools for the treatment of TMA-induced CHS.

Keywords: allergic contact, dermatitis; eosinophils; immunoglobulin E; neutrophils; trimellitic anhydride; tumor necrosis factor

Introduction

The contact hypersensitivity (CHS) response in mice is a model of clinical allergic contact dermatitis and also widely employed to investigate mechanisms of T lymphocyte-mediated inflammation (Grabbe and Schwarz, 1998; Kim *et al.,* 2003). Studies have demonstrated that local release of cytokines, including TNF-α, IL-1, and IFN-γ, is critical for the generation of the CHS reaction (Piguet *et al.,* 1991; Kondo *et al.,* 1995; Wang *et al.,* 2003).

TNF-α is a pleiotropic cytokine involved in host defense mechanisms and inflammatory responses (Vassalli, 1992; Shah *et al.,* 1995). TNF-α is produced in responses to various stimuli in a variety of cell types, such as mast cells, macrophages, monocytes, neutrophils, keratinocytes, and T cells (Hofsli *et al.,* 1988; Gordon and Galli, 1990; Ohkawara *et al.,* 1992; Bradding *et al.,* 1994). Neutralizing antibody against TNF-α inhibits the migration of epidermal Langerhans cells in response to hapten sensitization (Cumberbatch and Kimber, 1995). However, direct effect of TNF-α on the TMA-induced CHS in murine model is not fully understood.

Trimellitic anhydride (TMA), a sensitizer that induces occupational asthma, is widely used industrially to make epoxy and alkyd resins, plasticizers, high temperature polymer, and surfactants (Mapp *et al.,* 1999; Zhang *et al.,* 2002). Specific antibodies to TMA have been found in exposed workers, and TMA-induced occupational asthma is thought to be mediated by an IgE-mediated allergic mechanism. TMA also elicits eosinophil infiltration into lungs of sensitized mice (Zhang *et al.,* 2002). IgE antibodies mediate allergic diseases by binding to specific high

affinity receptors (FcεRI) on mast cells and basophils. Increased production of IgE in response to common environmental antigens is the hallmark of allergic diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis (Burr, 1993). Some studies showed the roles of IgE in the development of allergic inflammation, airway hyprresponsiveness, and contact hypersensitivity in a murine model (Haile *et al.,* 1999; Tumas *et al.,* 2001; Yokozeki *et al.,* 2003; Coyle *et al.,* 2005). However, the detailed role of IgE remains to be characterized.

Therefore, this study has investigated to roles of TNF-α and IgE in the TMA-induced CHS using TNF-$\alpha^{-/-}$ mice and a neutralizing anti-TNF-α or IgE antibodies.

Materials and Methods

Animals

Balb/c male mice aged 6 weeks were purchased from Korean Damool Science (Daejeon, Chungnam, Korea). TNF-α knockout (B6; 129S-Tnf^{tm1Gkl}, TNF-$\alpha^{-/-}$) and original wild type (B6129SF2/J, TNF-$\alpha^{+/+}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Each experimental group consisted of 5 mice. All experiments were repeated at least 2 times with similar results. They were housed in a laminar flow cabinet and artificial lighting conditions with 12-h day/night cycle and had access to food and water *ad libitum*.

Chemicals

TMA was purchased from Sigma (MA). A mixture of acetone (Junsei):olive oil (Filippo berio, Italy) (4:1, v/v) was used as vehicle.

Induction of contact hypersensitivity

Mice were sensitized on shaved back skin with 50 mg TMA in a 4:1 acetone:olive oil solution (A/O) on day 0 and 12.5 mg TMA on day 7 under light anesthesia according to method described by Lauerma *et al.,* (1997). On days 14, 21, 28 and/or 35, left ears were repeatedly challenged with 2 mg TMA and right ears were repeatedly challenged with 20 μl of A/O only (Figure 1).

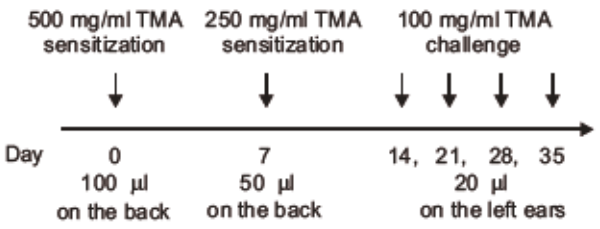


Figure 1. Schematic diagram of the experimental protocol. Mice were sensitized on shaved back skin with 100 μl of 500 mg/ml TMA on day 0 and 50 μl of 250 mg/ml TMA in A/O on day 7. On days 14, 21, 28, and 35, left ears were challenged with 20 μl of 100 mg/ml TMA and right ears were applied with 20 μl of A/O.

Treatment of anti-TNF-α or anti-IgE antibodies

An anti-TNF-α antibody (BD Biosciences, CA; 0.2, 2 or 5 mg/mouse) and control saline were injected into the peritoneal cavity 5 min before 100 mg/ml TMA challenge on day 21 (Choi *et al.,* 2003). An anti-IgE antibody (BD Biosciences, CA; 0.2, 2 or 20 mg/ mouse) and control saline were injected 24 h before 100 mg/ml TMA challenge on day 14 (Haile *et al.,* 1999).

Measurement of ear swelling responses (ESR)

The ear thickness just before and after the last challenge was measured three times with a dial thickness gauge (Model 7326, Tokyo, Japan). In the time-course study, the ear thickness was measured 1, 2, 4, 8, 12, 24, 48, and 72 h after challenge. Ear thickness was expressed in units of 10^{-4} inches (mean ± SEM). Ear swelling responses (ESR) were calculated as the following formula.

ESR = [ear thickness after TMA challenge − ear thickness before TMA challenge]

Histological examination

Seventy-two hours after the last challenge, animals were sacrificed and both ears of animals were excised. Specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 4 μm section by microtome (SM 2000R, Leica, Germany; Lee *et al.,* 2004). The sections were stained with Wright Giemsa solution for polymorpholeukocytes (Stern *et al.,* 1997) and Congo red solution for eosinophils (Song *et al.,* 1999).

Statistical analysis

Data were presented as mean ± standard error of mean (SEM). Student's unpaired *t*-test was applied to determine significant differences between corresponding treated groups and control groups. A value of $P < 0.05$ was considered statistically significant.

Results

Trimellitic anhydride induces the biphasic increase in ESR

Time course of the ESR to the TMA challenges are shown in Figure 2. First challenge of TMA resulted in

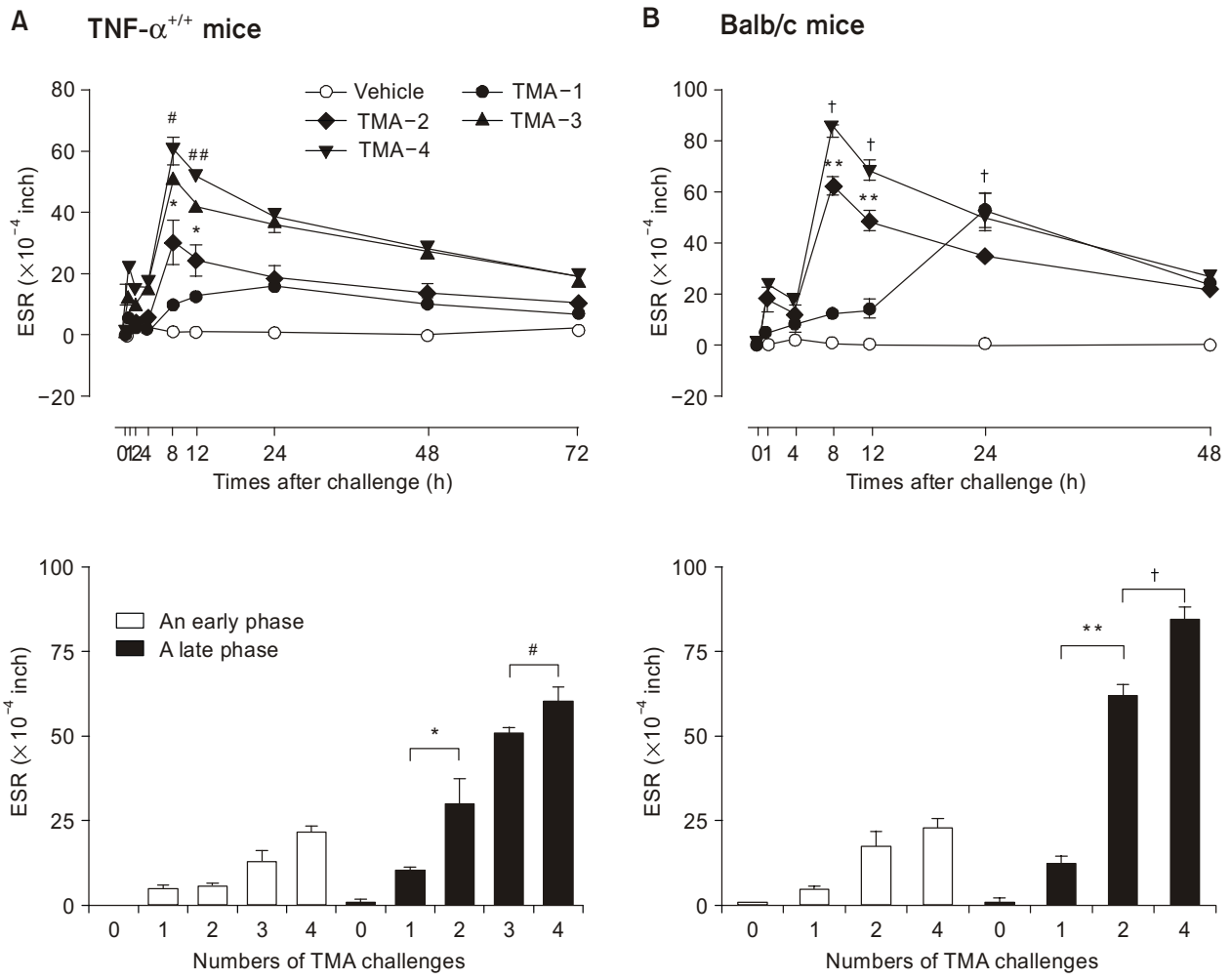


Figure 2. ESR on time course following TMA challenges on the ears of the TNF-$\alpha^{+/+}$ (B6129SF2/J) (A) and Balb/c (B) mice sensitized on the back skin with TMA. Differences from TMA-1, **P* < 0.01; ***P* < 0.001, from TMA-2, $^{\dagger}P$ < 0.01 and from TMA-3, $^{\#}P$ < 0.01, $^{\#\#}P$ < 0.001

an increase of the ESR of both Balb/c and TNF-$\alpha^{+/+}$ mice at 1 h and 24 h after the challenge, showing a biphasic response. However, the late phase of ESR was significantly greater than the early phase. The late phase response in Balb/c was approximately 1.5-fold greater than in TNF-$\alpha^{+/+}$ mice, suggesting that there are species differences in TMA-induced CHS. Vehicle challenge showed no effects on the ESR. Increase in the number of TMA challenges resulted in the increase of ESR in a challenge number-dependent manner, suggesting that this may not be only cumulative effect of TMA. The late phase of CHS was observed in both mice groups at 8 h after TMA challenges. These results indicate that mice sensitized to TMA could possibly offer a useful model to study the mechanism of CHS.

Depletion of TNF-α attenuates the late phase of ESR

To evaluate whether TNF-α plays a role in developing CHS, we examined the TMA-induced ESR using TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. Time courses of ESR to TMA challenge are shown in Figure 3. First challenge of TMA increased the ESR of both TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice at 1 h and 24 h after the challenge, showing a biphasic response. Vehicle challenge showed no effects on the ESR of both TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. Increase in the number of TMA challenges resulted in the increase of ESR in a challenge number-dependent manner. The late phase of CHS was observed in both mice groups at 8 h after TMA challenges. The magnitude of these responses observed according to the frequency of the TMA challenge in TNF-$\alpha^{+/+}$ mice was significantly higher than that in TNF-$\alpha^{-/-}$ mice. In the group of 4th

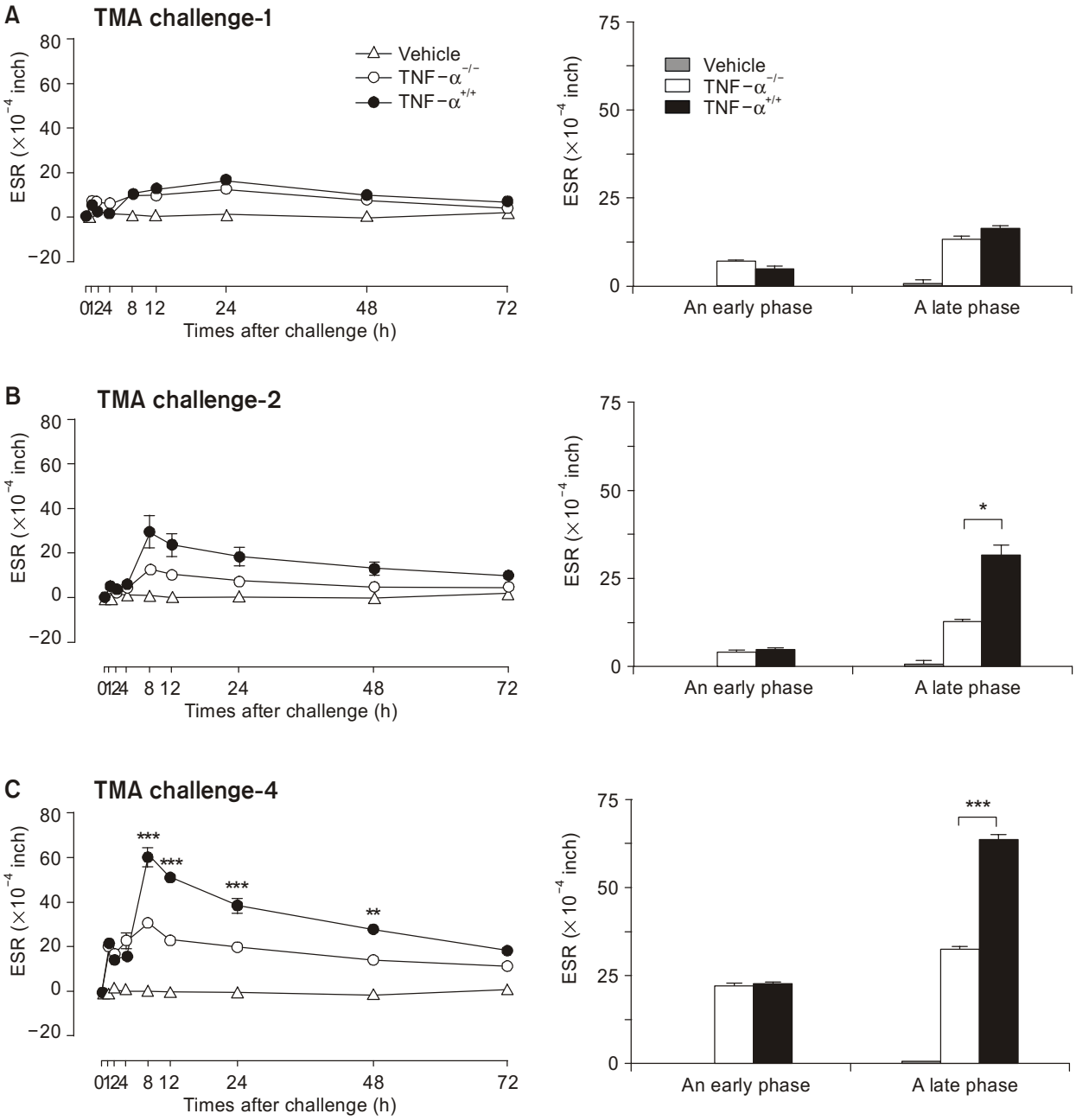
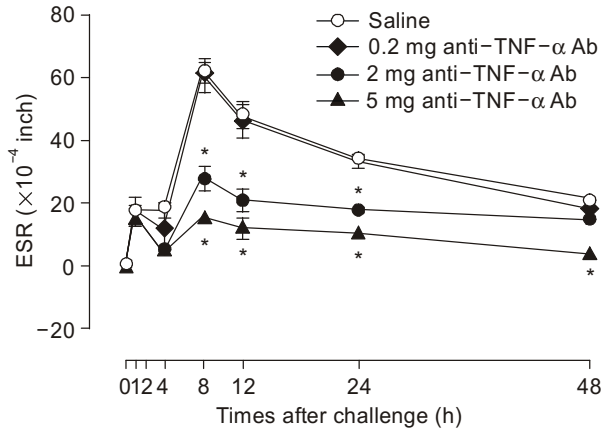


Figure 3. ESR on time course following once (A), twice (B) and four times (C) of TMA challenge on the ears of the TNF-α knockout TNF-$\alpha^{-/-}$ (B6;129S-Tnf^{tm1Gk1}, open circle) and TNF-$\alpha^{+/+}$ (B6129SF2/J, closed circle) mice sensitized on the back skin with TMA. Differences from TNF-$\alpha^{-/-}$, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

challenge, the late phase of CHS in TNF-$\alpha^{-/-}$ mice showed approximately 2.5-fold decrease compared to that in TNF-$\alpha^{+/+}$ mice. These data indicate that TNF-α is a key cytokine involved in the development of the late phase of CHS.

Inhibition of TNF-α attenuates the late phase of ESR

To identify the role of TNF-α in developing CHS, we examined the TMA-induced ESR using Balb/c injected anti-TNF-α antibody into the peritoneal cavity at 5 min before the second TMA challenge. Pretreatment of anti-TNF-α antibody inhibited the TMA-induced late phase of ESR in a dose-dependent manner, but anti-TNF-α antibody did not attenuate the early phase of ESR (Figure 4). Pretreatment of saline did not inhibit the ESR. The late phase of



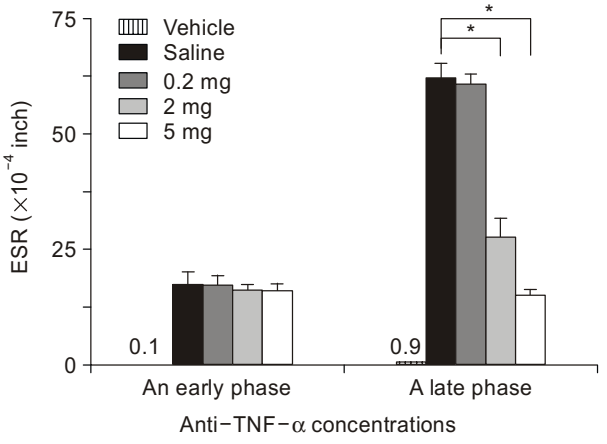
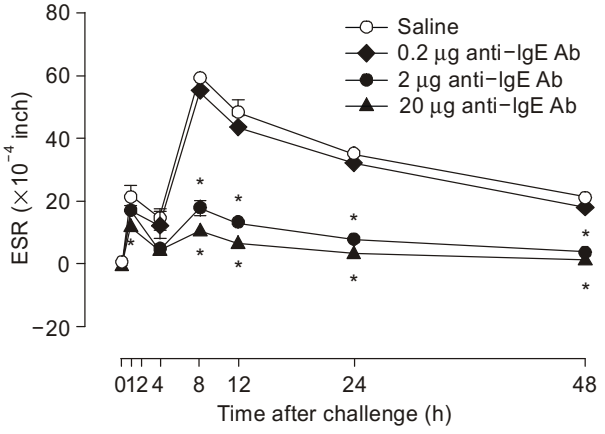


Figure 4. Effect of anti-TNF-α antibody on the TMA-induced ear swelling responses (ESR) in the Balb/c mice. The sensitized mice were challenged with 100 mg/ml TMA to the left ears on day 14. On day 21, saline or anti-TNF-α antibody was injected into the peritoneal cavity at 5 min before the second TMA challenge. Differences from saline, **P* < 0.001.



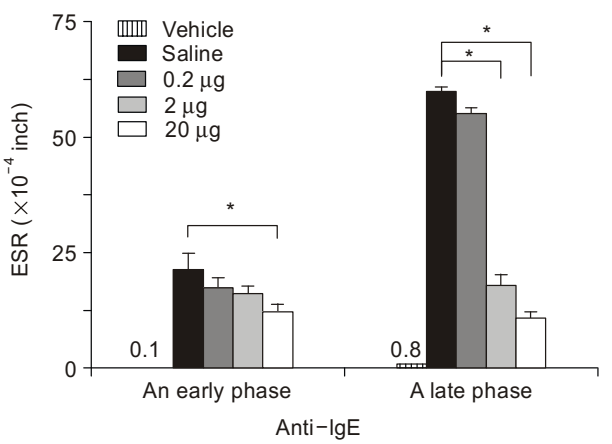


Figure 5. Effect of anti-IgE antibody on the TMA-induced ear swelling responses (ESR) in the Balb/c mice. The sensitized mice were challenged with 100 mg/ml TMA to the left ears on day 14. On day 14, saline or anti-IgE antibody was injected subcutaneously at 24 h before the first TMA challenge. Differences from saline, **P* < 0.001.

ESR in anti-TNF-α antibody-injected group was approximately 3.5-fold lower than in saline-injected control group. These results strongly suggest that the increase of late phase of CHS by repeated TMA challenge may depend on the TNF-α at the site of inflammation.

Inhibition of IgE attenuates the late phase of ESR

To evaluate whether IgE plays a role in developing CHS, we examined the TMA-induced ESR using Balb/c subcutaneously injected anti-IgE antibody at 24 h before the first TMA challenge. Pretreatment of anti-IgE antibody inhibited the TMA-induced late phase of ESR in a dose-dependent manner, and at a concentration of 20 μg/mouse, anti-IgE antibody almost completely attenuated the early phase of ESR (Figure 5). Pretreatment of saline did not inhibit the ESR. The late phase response in anti-IgE antibody-injected group was approximately 4-fold lower than in saline-injected control group. These results strongly suggest that IgE is associated with the late phase responses of repeated TMA challenge.

Inhibition of TNF-α or IgE attenuates trimellitic anhydride-induced leukocyte infiltration into the dermis of the ears

The infiltration of polymorphonuclear leukocytes (PMNLs) and eosinophils were determined as a cellular mechanism underlying ESR. As shown Figures 6 and 7, TMA challenge resulted in an induction of the significant infiltrations of PMNLs and

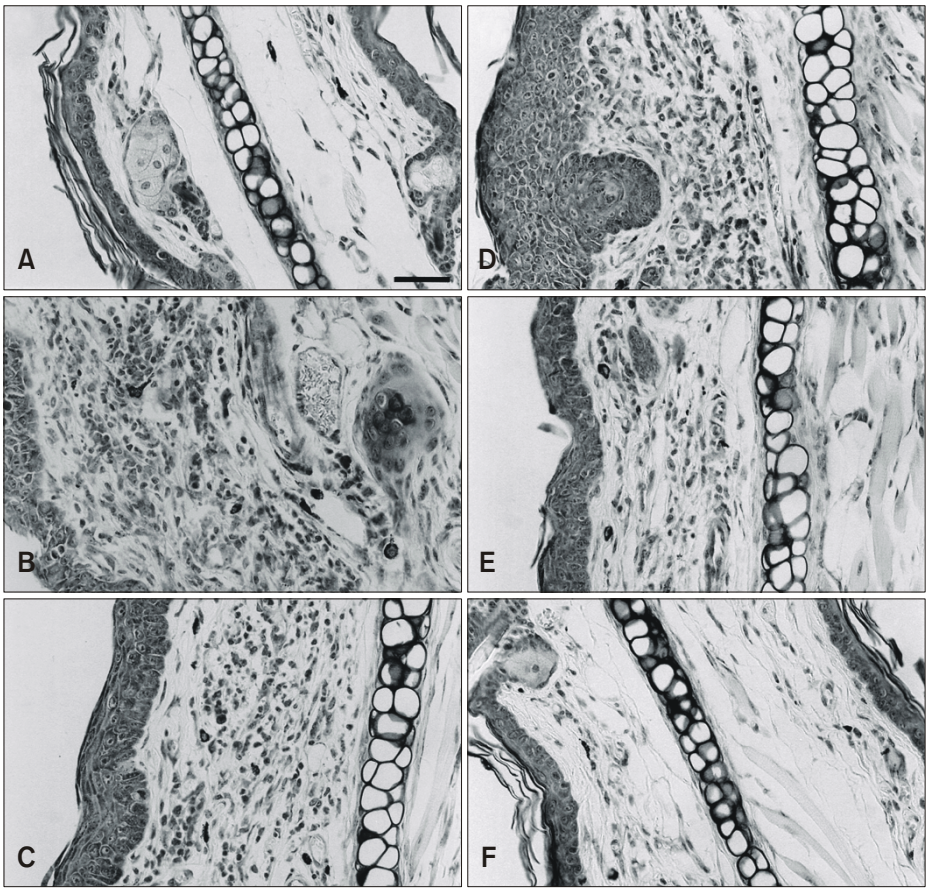


Figure 6. Light micrographs of the infiltration of polymorphonuclear leukocytes by repeated TMA challenges from the control (vehicle treated, A), TNF-$\alpha^{+/+}$ (B6129SF2/J, B) and TNF-α knockout TNF-$\alpha^{-/-}$ (B6;129S-Tnf^{tm1Gk1}, C) mice sensitized on the back skin with TMA. The sensitized Balb/c mice were challenged with 100 mg/ml TMA to the left ears on day 14. On day 21, saline (D) or anti-TNF-α antibody (5 mg/mouse, E) was injected into the peritoneal cavity at 5 min before the second TMA challenge. On day 14, anti-IgE antibody (20 μg/mouse, F) was injected subcutaneously at 24 h before the first TMA challenge. Wright Giemsa stain, Bar = 50 μm.

eosinophils into the dermis of the ears in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. However, the extent of infiltrations of PMNLs and eosinophils observed in TNF-$\alpha^{-/-}$ mice was significantly lower than that in TNF-$\alpha^{+/+}$ mice. The number of PMNLs and eosinophils per unit area in the ears of TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice were shown in Table 1.

To assess the role of TNF-α in developing CHS, we examined the TMA-induced leukocyte infiltration using Balb/c injected with anti-TNF-α antibody into the peritoneal cavity at 5 min before the second TMA challenge. As shown Figures 6 and 7, TMA challenge induced the infiltrations of PMNLs and eosinophils into the dermis of the ears in saline-treated control mice. However, treatment of anti-TNF-α antibody significantly inhibited the infiltrations of PMNLs and eosinophils.

To examine the role of IgE in the late phase of CHS, we determined the TMA-induced leukocyte infiltration using Balb/c subcutaneously injected anti-IgE antibody at 24 h before the first TMA challenge. As shown Figures 5 and 6, TMA challenge induced the infiltrations of PMNLs and eosinophils into the dermis of the ears in saline-treated control mice. Treatment of anti-IgE antibody significantly inhibited the infiltrations of PMNLs and eosinophils in a dose-dependent manner. Table 2 shows that the number of PMNLs and eosinophils per unit area in the ears of saline-, anti-TNF-α antibody- or anti-IgE antibody-injected mice. These data indicate that TNF-α or IgE are important modulators involved in the development of the late phase of CHS.

Table 1. The number of polymorphonuclear leukocytes (PMNLs) and eosinophils (Eos) per unit area (mm^2) on 72 h after four repeated TMA challenges in the ears of TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice.

Groups	No. of PMNLs[a)]	No. of Eos[b)]
TNF-$\alpha^{+/+}$	584.3 ± 109	249.0 ± 81.3
TNF-$\alpha^{-/-}$	320.8 ± 99.7*	113.5 ± 39.8*

[a)]The 4 μm-thickness paraffin sections were stained with Wright Giemsa solution. *Difference from TNF-$\alpha^{+/+}$, $P < 0.05$. [b]The 4 μm-thickness paraffin sections were stained with Congo red solution. *Difference from TNF-$\alpha^{+/+}$, $P < 0.05$.

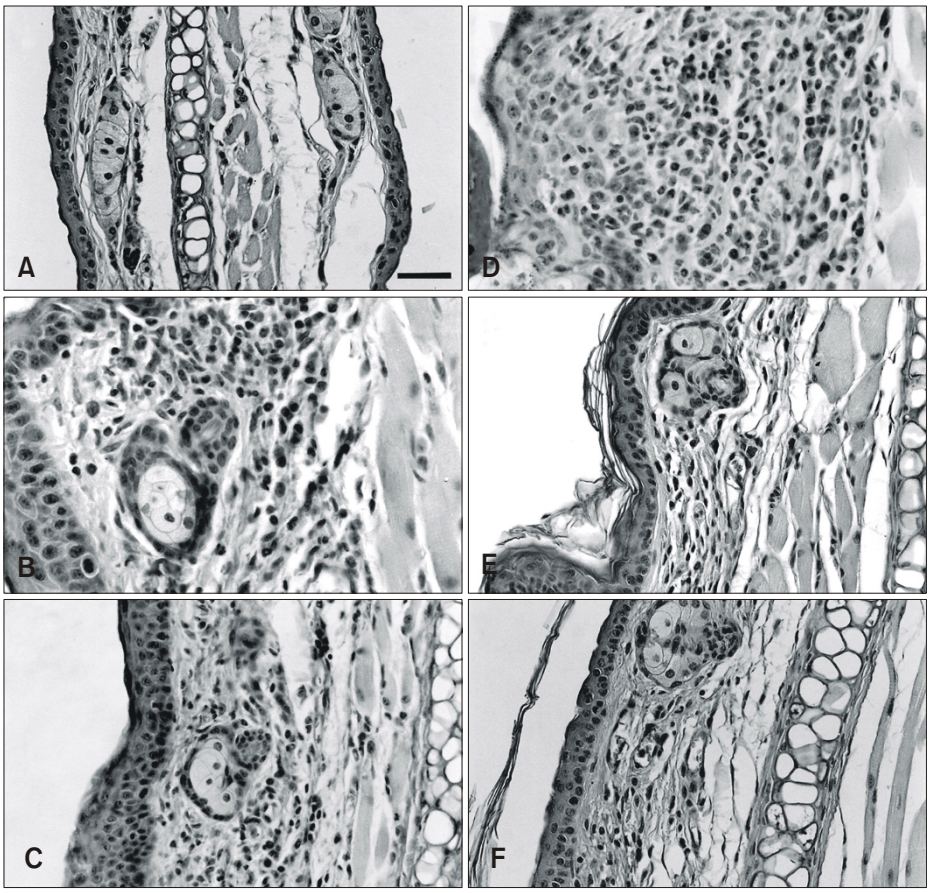


Figure 7. Light micrographs of the infiltration of eosinophils by repeated TMA challenges from the control (vehicle treated, A), TNF-$\alpha^{+/+}$ (B6129SF2/J, B) and TNF-α knockout TNF-$\alpha^{-/-}$ (B6;129S-Tnf^{tm1Gk1}, C) mice sensitized on the back skin with TMA. The sensitized Balb/c mice were challenged with 100 mg/ml TMA to the left ears on day 14. On day 21, saline (D) or anti-TNF-α antibody (5 mg/mouse, E) was injected into the peritoneal cavity at 5 min before the second TMA challenge. On day 14, anti-IgE antibody (20 μg/mouse, F) was injected subcutaneously at 24 h before the first TMA challenge. Congo red stain, Bar = 50 μm.

Table 2. The number of polymorphonuclear leukocytes (PMNLs) and eosinophils (Eos) per unit area (mm^2) on 48 h after two repeated TMA challenges in the ears of Balb/c mice.

Groups[a)]	No. of PMNLs[b)]	No. of Eos[c)]
Saline	792.8 ± 110	345.6 ± 56.13
5 mg of anti-TNF-α Ab	268.6 ± 30.1*	142.4 ± 21.4*
20 μg of anti-IgE Ab	153.1 ± 15.9*	85.1 ± 10.8*

[a)]The sensitized mice were challenged with 100 mg/ml TMA to the left ears on day 14. On day 21, anti-TNF-α antibody was injected into the peritoneal cavity at 5 min before the second TMA challenge. Anti-IgE antibodies were injected 24 h before 100 mg/ml TMA challenge on day 14. In control group, saline instead of anti-TNF-α antibody or anti-IgE was injected into the peritoneal cavity or subcutaneously. [b)]See the foot note of Table 1-a. *Difference from saline, $P < 0.05$. [c)]See the foot note of Table 1-b. *Difference from saline, $P < 0.05$.

Discussion

In 1935, Landsteiner and Jacobs showed that epicutaneous application of small reactive compounds results in the induction of CHS (Landsteiner and Jacobs, 1935). The first treatment (sensitization) had no visible effect, but when the same hapten was applied a second time (elicitation) after a week, a local inflammation at the site of application occurs with a delay of 24-48 h.

In this study, we have found that the repeated challenges of TMA induced biphasic ESR, an early and a late phase responses. The repetition of the challenge enlarged the extent of an early and a late phase of CHS in TNF-$\alpha^{+/+}$ (B6129SF2/J) and Balb/c mice. In the late phase of TMA-induced CHS, the peak of ESR by single challenge showed at 24 h after challenge, whereas the peak was observed at 8 h after repeated challenge. At the repeatedly TMA-challenged skin sites, the number of dermal PMNLs and eosinophils was increased. Dilatation of blood vessels in the dermis of skin was observed. Plugs of PMNLs and eosinophils in the ears were also observed in stratum corneum of the epidermis. The morphologic studies on the delayed type CHS in human have reported a number of important features to characterize these reactions (Dvorak *et al.,* 1974; 1976a; 1976b). These include infiltration and

piecemeal degranulation of basophils, degranulation and replication of fixed tissue mast cells, infiltration of eosinophils and neutrophils, increased vascular permeability leading to dermal and epidermal edema, vascular compaction, and erythrocyte extravasation; microvascular alterations affecting endothelial cells and pericytes, with compromise of vessel lumina and basement membrane thickening. Histological examination has also revealed the infiltration of basophils and eosinophils in the superficial dermis as the characteristics of the animal skin reaction (Sugawara *et al.,* 1993).

Studies have demonstrated that the local release of cytokines, including TNF-α, IL-1, and IFN-γ, is critical for the optimal generation of the CHS and that TNF-α is believed to play a role in the pathogenesis of asthma in humans and asthma and CHS in animal models (Piguet *et al.,* 1991; Kondo *et al.,* 1995; Wang *et al.,* 2003). TNF-α, which is recognized as a powerful mediator of inflammatory reaction, might induce these alterations either directly, since it can react with a wide range of cell types (Beutler and Cerami, 1989), or indirectly by the activation of several other cells. Our study has revealed that the extent of ESR observed in TNF-$\alpha^{-/-}$ mice was significantly lower than that in TNF-$\alpha^{+/+}$ mice. Treatment of anti-TNF-α antibody decreases the magnitude of a late phase response of CHS. Histologically, infiltration of PMNLs and eosinophils increased in repeatedly TMA-challenged the TNF-$\alpha^{-/-}$ mice, but the extent of the infiltration is decreased as compared to that in TNF-$\alpha^{+/+}$ mice. Administration of anti-TNF-α antibody also decreased significantly the infiltration of PMNLs and eosinophils into the dermis of TMA-challenged Balb/c mice. Our data are in agree with the report of Nakae and colleague (2003), which showed that CHS is suppressed by the injection of anti-TNF-α antibody. Oxazolone-induced CHS is reduced in TNF-$\alpha^{-/-}$ and TNF receptor II$^{-/-}$ mice (Pasparakis *et al.,* 1996; Wang *et al.,* 1997). Piguet and colleague (1991) have reported three lines of evidence that argue in favor of a major role of TNF-α in both irritant reaction and CHS; first, these reactions are associated with a marked rise in the TNF-α mRNA level; second, they are considerably reduced or abrogated by treatment with anti-TNF-α antibody; third, a continuous hypodermal infusion of TNF can reproduce many of the dermal or epidermal features of CHS, notably epidermal necrosis, dermal leukocytic infiltration, and hemorrhagic necrosis (Piguet *et al.,* 1990). Treatment of anti-TNF-α antibody prevents the various features of the CHS, as seen on histological sections, e.g., leukocyte infiltration and hemorrhages within the dermis and keratinocytes necrosis. It has been demonstrated that TNF-α mRNA is detectable in an untreated ear, increases after the application of trinitrochlorobenzene in nonsensitized mice, and is highest in sensitized mice. A study has reported that TNF-α mRNA accumulation, which is evident 0.5 h after hapten application, is abolished by the treatment with anti-TNF-α antibody (Piguet *et al.,* 1991).

Although an involvement of other cytokines such as IL-1and IL-4 in CHS development is only obvious under conditions using specific allergens or specific genetic backgrounds (Nagai *et al.,* 2000; Nakae *et al.,* 2003), it is clearly observed using TMA as contact allergen that TNF-α for CHS development is a critical factor.

IgE antibodies mediate allergic diseases by binding to specific high affinity receptors (FcεRI) on mast cells and basophils. An increase in production of IgE in response to common environmental antigens is the hallmark of allergic diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis (Burr, 1993). There is an evidence for the importance of IgE in the development of bronchial asthma, in that studies have demonstrated a correlation between IgE serum levels and the prevalence (Burrows *et al.,* 1989) or severity (Sears *et al.,* 1991). Therefore, the IgE/FcεRI interaction is a target for clinical intervention in allergic diseases. Yokozeki and colleague have demonstrated that Th2 cytokines (IL-4 and IL-5) and IgE as well as mast cells play an essential role in the induction of para-phenylenediamine-induced CHS (2003). Our results show that treatment of anti-IgE antibody decreases the magnitude of a late phase of CHS. Histologically, anti-IgE antibody also significantly reduces infiltration of PMNLs and eosinophils into the dermis of TMA-challenged Balb/c mice. Our data are in agree with revious reports, which show that IgE plays a critical role in the production of Th2 cytokienes in the development of eosinophil recruitment into the inflammation site after antigen provocation and the initial development of the late allergic responses (Haile *et al.,* 1999; Tumas *et al.,* 2001; Yokozeki *et al.,* 2003; Coyle *et al.,* 2005).

In summary, TMA can be used to establish a murine model of CHS, and TNF-α and IgE may act as potential modulators in the TMA-induced CHS. Neutralization of TNF-α and IgE by anti-TNF-α or anti-IgE antibodies may provide a therapeutic tool for the treatment of TMA-induced CHS.

Acknowledgement

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