

Epicutaneous administration of hapten through patch application augments TH2 responses which can downregulate the elicitation of murine contact hypersensitivity

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Summary

Background and objective Allergic contact dermatitis and its animal model, contact hypersensitivity (CHS), have long been documented as type 1 T-cell-predominant immune responses. Although type 1/type 2 T-cell deviation has been repeatedly demonstrated to play an important role in many human diseases and their animal models, the potential of tilting type 1/type 2 T-cell differentiation of CHS by modulating the manner of administration and dosage of hapten remains unexplored. This study examined the effect of these two factors on type 1/type 2 balance of CHS.

Methods ELISA methods for detection of isotypes of hapten-specific antibodies and cytokine profiles of *in vitro* reactivation culture as well as ear-swelling assay were used to indicate type 1 or type 2 T-cell immune responses.

Results In this paper, it was demonstrated that dosage of hapten has no effect on the type 1/type 2 T-cell balance of CHS, whereas epicutaneous administration of hapten through patch application could tilt the type 1/type 2 balance to decrease type 1 and to augment type 2 T-cell responses. Patch application-induced modulation is still effective in ever-sensitized mice and the augmented type 2 T-cell responses are persistent and increase progressively in strength after repeated immunizations. Moreover, it was demonstrated that the augmented type 2 T-cell response can downregulate the elicitation of CHS. The major mediating cells of the enhanced type 2 T-cell responses were determined to be CD4⁺ T cells (TH2 cells). **Conclusions** These data show that epicutaneous administration of hapten through patch application augments TH2 response which can downregulate the elicitation of murine CHS. This exploration may contribute to the understanding of regulatory mechanisms involved in contact allergy.

Keywords: contact hypersensitivity, hapten, patch application, T-cell deviation

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Introduction

Two subsets of CD4⁺ T helper (TH) cells with different lymphokine production profiles and functions were first identified in the 1980s [1]. TH1 and TH2 cells provide help for cellular and humoral immunity, respectively, and they regulate reciprocally. Thereafter, similar subpopulations

were observed in CD8⁺ cytotoxic T (Tc) cells (Tc1 and Tc2) as well [2]. Accumulated evidence indicated that type 1/type 2 T-cell deviation plays a very important role in the pathogenesis of many human diseases [3,4]. Many efforts have been made to manipulate the type 1/type 2 T-cell differentiation with the hope that this strategy could be applied to the treatment and prevention of human disease [5]. Allergic contact dermatitis and its murine model (contact hypersensitivity; CHS) have long been documented as type 1 (TH1 and/or Tc1)-predominant T-cell immune responses [6,7]. Yet recent reports from several laboratories

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have shown that type 2 T-cell responses are also induced in human disease states and CHS animal models [8–12]. However, studies aimed at defining the functions of the type 2 T-cell response in the CHS model yielded conflicting results [13–19]. Some studies indicated that type 2 T-cell immune responses have contributed to CHS [18,19], whereas others showed that type 2 T-cell immune responses have a negative regulatory role in CHS [13–17]. Weigmann *et al.* tried to reconcile these data by the hypothesis that IL-4 has a dual effect on the elicitation phase of CHS: inhibitory at a high concentration vs enhancing at a low concentration [19].

It was demonstrated that epicutaneous administration of protein antigen through patch application induces a predominant TH2 response with high IgE production in mice [20]. Several possibilities exist to explain the discrepancy between these observations and the classical type 1 T cell-predominant CHS model, including antigen character, antigen dose and manner of administration. The purpose of the current study was to investigate the effects of manner of administration and dosage of hapten on type 1/type 2 balance of hapten-induced immune response. It was demonstrated that dosage of hapten has no effect on type 1/type 2 T-cell differentiation, whereas administration of hapten through patch application augments type 2 T-cell responses which could negatively regulate the type 1-predominant CHS.

Materials and methods

Mice and haptens

Six- to 10-week-old female BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University. The haptens used in this study were 2,4,6-trinitrochlorobenzene (TNCB, TCI, Tokyo) and 2,4,6-trinitrobenzene-sulfonic acid hydrate (TNBS, Sigma, St Louis, MO, USA).

Immunization

Two methods were used for immunization: traditional painting and patch application. For traditional painting, mice were sensitized by painting 50 μ L of various concentrations of TNCB acetone/olive oil (4:1) solution on the shaved abdomen. For patch application, 100 μ L of various concentrations of TNBS water solution were first applied to 1 cm² gauze on the patches, which were then applied to the shaved back skin, then fixed and secured with an elastic bandage. Patches were left on for 3 days and freshly prepared patches were reapplied for another 3 days. For second and repeated immunization, similar procedures were performed in 3 week intervals.

Determination of TNP-specific Ab levels

One week after each immunization, mice were bled and sera were prepared for determination of TNP-specific IgG2a and IgE levels. Conventional protocols [21] including TNP-ovalbumin conjugate and isotype-specific secondary antibodies (Abs) were used. An ELISA amplification system (Life Technologies, Gaithersburg, MD, USA) was included in IgE determination to improve the sensitivity. Ab titres were calculated based on linear regression analysis of the absorbances. Results are expressed as ELISA titres relative to an internal isotype standard run in each assay.

Measurement of IFN- γ and IL-4 secretion

One week after primary immunization, three mice per experimental group were killed to obtain axillary, subscapular and inguinal lymph nodes. Lymph node cells (LNCs) were incubated at 37°C for 1 h to remove adherent cells. Spleen cells from naive mice were pulsed with TNP by incubation with 10 mmol/L TNBS at 37°C for 10 min. TNP-pulsed and -unpulsed irradiated spleen cells (4×10^5) and LNCs (2×10^5) were co-cultured in U-bottomed 96-well plates. After 48 h, supernatants were collected and the amounts of IFN- γ and IL-4 secretion were determined by ELISA kits from Endogen (Boston, MA, USA). For Ab blocking assay, anti-CD4 Ab and anti-CD8 Ab were added to the culture system in a final concentration of 1 μ g/mL.

Ear swelling assay

Five days after immunization, mice were challenged by applying 20 μ L 1% TNCB solution in 9:1 olive oil/acetone to each animal's left ear (10 μ L for each side). The increase in ear thickness was measured with an engineer's micrometer before and 24 h after challenge and expressed in units of 10^{-2} mm $\pm$ SD. Mice challenged without prior immunization served as negative controls. Each treatment group consisted of five mice, and each experiment was performed at least twice.

Adoptive transfer

Recipient mice (five per group) were immunized by traditional painting (6 mg of TNCB) and challenged as described in the ear swelling assay. Then 4×10^7 draining LNCs — derived from donor mice ($n = 12$ for total lymph node cell transfer, $n = 22$ for adoptive transfer after CD4⁺ or CD8⁺ T-cell depletion) five days after primary immunization by traditional painting (6 mg of TNCB) or patch application (3 mg of TNBS, twice) — were suspended in 0.25 mL of

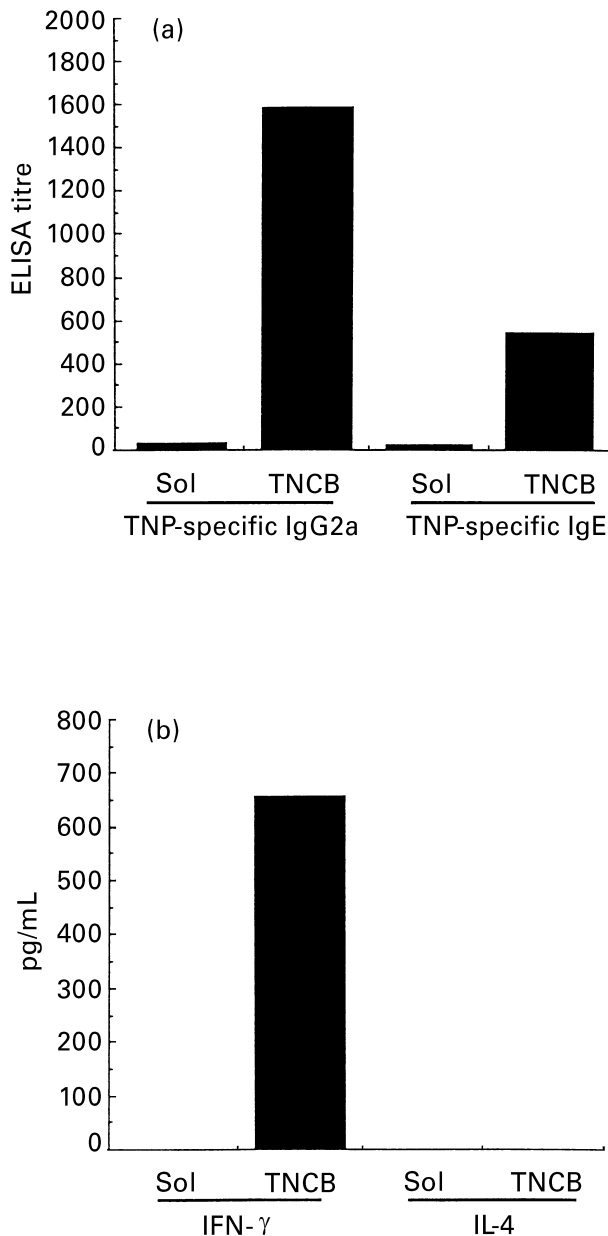


Fig. 1. Type 1 T-cell immune responses dominate in TNP-induced classical CHS model with concurrent marginal type 2 T-cell responses. (a) BALB/c mice (eight per group) were immunized twice with 6 mg TNCB in acetone/olive oil solution, or solvent only, by traditional painting over a 3-week interval. Sera were collected 1 week after re-immunization. Levels of TNP-specific IgG2a and IgE were determined by ELISA. (b) BALB/c mice (three per group) were immunized once as for (a). One week later, draining lymph nodes were dissected and pooled LNCs (2×10^6 /well) were co-cultured with irradiated, TNP-modified spleen cells from naive BALB/c mice (4×10^6 /well) in U-bottom 96-well plates. Supernatants were collected at 48 h. IFN- γ and IL-4 contents were determined by ELISA kits. Results are from one representative experiment out of two performed.

HBSS and injected i.v. into each recipient mouse 1 h before sensitization or challenge. For depletion of CD4⁺ or CD8⁺ T cells from immunized LNCs, the panning method was used. Briefly, 75 μ g anti-CD4 Ab or anti-CD8 Ab were added to 5 mL sterile PBS in culture plates (Corning, NY, USA) and incubated at 4°C overnight. After blocking with culture medium at 37°C for 1 h, 5×10^7 suspended LNCs were added and incubated at room temperature for another hour. Unbound cells were collected by gently shaking the plates. The yield after CD4⁺ T cell-depletion and CD8⁺ T cell-depletion were 31–35% and 67–72%, respectively. The purity of CD4⁺ or CD8⁺ T cells was higher than 93% in all experiments.

Results

Type 1 T-cell immune responses dominate in TNP-induced classical CHS model with concurrent marginal type 2 T-cell responses

First type 1/type 2 balance was investigated in the classical CHS model. Two indicators were chosen to represent type 1 T-cell response: serum levels of hapten-specific IgG2a [22], and the amount of IFN- γ secretion in *in vitro* reactivation of LNCs derived from hapten-primed mice. In contrast, serum levels of hapten-specific IgE and IL-4 secretion represented type 2 T-cell immune responses. Groups of BALB/c mice were immunized twice in a 3-week interval by traditional painting with 6 mg TNCB in acetone/olive oil solution. One week after the second immunization, sera were collected and levels of TNP-specific IgG2a and IgE were each determined. As shown in Fig. 1a, a high level of TNP-specific IgG2a and low level of TNP-specific IgE could be detected. Another group of BALB/c mice were immunized by painting, and the content of IFN- γ and IL-4 in the supernatants of *in vitro* reactivation culture of draining LNCs were measured. Figure 1b showed that a significant amount of IFN- γ could be detected in the culture supernatant, whereas the IL-4 content was below detection sensitivity (<2.5 pg/mL). These results indicate that epicutaneous administration of hapten through traditional painting induces predominant type 1 T-cell responses with concurrent marginal type 2 T-cell responses.

Influence of the manner of administration and antigen dosage on type 1/type 2 T-cell development of hapten-specific immune responses

Two well-recognized factors which could be manipulated to control type 1/type 2 T-cell development are the manner of administration and the dosage of antigen. To determine the effect of these two factors on hapten-specific immune responses, groups of BALB/c mice were immunized twice

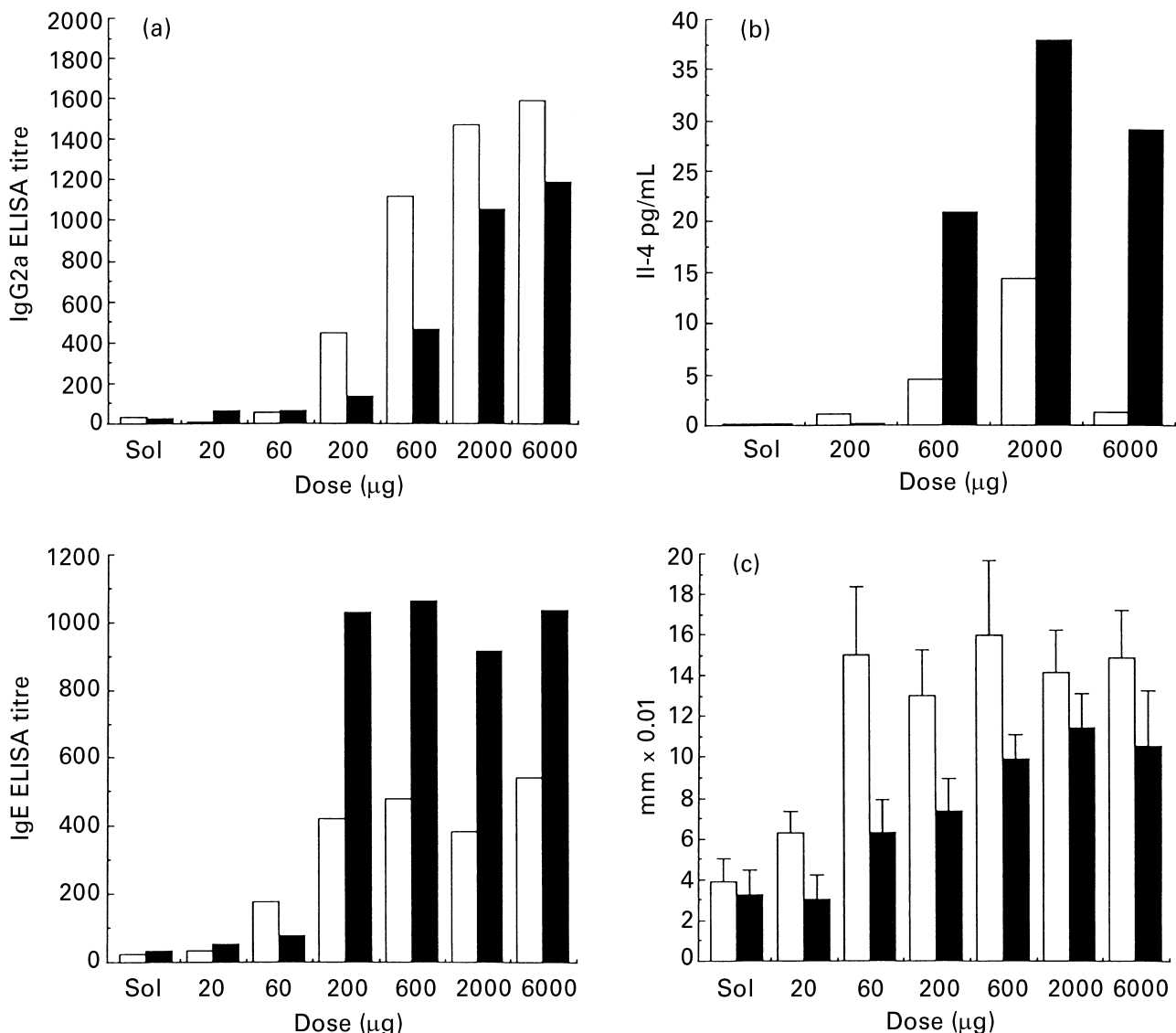


Fig. 2. Analysis of type 1/type 2 T-cell differentiation induced by epicutaneous exposure of hapten by modulating the manner of administration and dosage. BALB/c mice immunized with various doses of hapten through traditional painting (open bar) or patch application (filled bar). The marked doses represent the total doses of hapten administered in each immunization. (a) Mice (six to 10 per group) were immunized twice at a 3-week intervals. One week after second immunization, sera were collected. Levels of TNP-specific IgG2a and IgE were determined by ELISA. Geometric means of each group are presented. (b) Mice (three per group) were immunized. One week after immunization, draining lymph nodes were excised. Pooled LNCs were incubated at 37 °C for 1 h to remove adherent cells, thereafter, co-cultured (2×10^6 /well) with irradiated, TNP-modified spleen cells (4×10^6 /well) which had been incubated with 10 mmol/L TNBS at 37 °C for 10 min, in 96-well U-bottom plates. At 48 h, supernatants were collected and the amounts of IL-4 secretion were determined by ELISA kit from Endogen. (c) Mice (five per group) were immunized. Five days after immunization, mice were challenged with 20 µL 1% TNCS in olive oil/acetone (9:1) solution applied onto the left ear (10 µL for each side). The increase (mm $\times$ 0.01) in ear thickness was measured with an engineer's micrometer before and 24 h after challenge. The results were from one representative experiment out of two performed.

by epicutaneous exposure of various doses of hapten administered through either traditional painting or patch application. Sera were collected one week after second immunization to determine seral levels of hapten-specific IgG2a and IgE. Figure 2a shows that the levels of

TNP-specific IgG2a of mice receiving traditional painting were always higher than those for mice receiving patch application. On the contrary, the levels of TNP-specific IgE of mice receiving traditional painting were always lower than those mice receiving patch application. Moreover,

mice receiving various doses of hapten (20 µg to 6 mg) seemed to have similar IgG2a/IgE response patterns. The type 1/type 2 differentiation in the cytokine profile of *in vitro* reactivation culture system of draining lymph node cells was then examined. As shown in Fig. 2b, LNCs derived from patch-applied mice always produced higher amounts of IL-4 than those from traditional painting. The contents of IFN-γ were generally similar in groups of traditional painting and patch application (data not shown). However, the amount of IFN-γ varied markedly in each experiment. A search was made for another indicator (ear swelling assay) to represent type 1 T-cell immune responses. Figure 2c showed that mice receiving traditional painting always developed stronger ear swelling responses compared with those receiving patch application. Thus, these experiments indicate that modulation of hapten dosage seems unable to influence the type 1/type 2 T-cell differentiation of hapten-induced immune response. However, epicutaneous administration of hapten through patch application could tilt the type 1/type 2 balance to decrease type 1 and augment type 2 T-cell response.

The type 2 T-cell response induced by patch application is persistent, progressively increasing in strength after repeated immunization

Examination was done of the persistence of the enhanced type 2 T-cell response induced by patch application after repeated immunization. Groups of BALB/c mice received repeated immunization through patch application at three-week intervals, and sera were collected one week after each immunization. As shown in Fig. 3, the levels of TNP-specific IgE progressively increased in titres after repeated immunization with all tested antigen doses. This result is encouraging in that a persistent, progressively stronger type 2 T-cell immune response could be induced by repeated immunization through patch application.

The patch application-induced modulation was still effective on ever-immunized mice

To evaluate the potential of patch application, its effect on type 1/type 2 balance was tested on ever-immunized, type 1-predominant mice. Groups of BALB/c mice were all sensitized by traditional painting. After three weeks the mice then received reimmunization through patch application or traditional painting. As shown in Fig. 4, the mice receiving reimmunization through patch application showed less ear swelling after challenge. Thus, the modulation of type 1/type 2 balance induced by patch application is still effective on ever-immunized mice.

The augmented type 2 T-cell response induced by patch application could negatively regulate CHS in the elicitation but not the sensitization phase

We further set up an adoptive transfer system to examine the biological effect of patch-enhanced type 2 T-cell response on type 1 T-cell predominant CHS. The recipient BALB/c mice were immunized by traditional painting

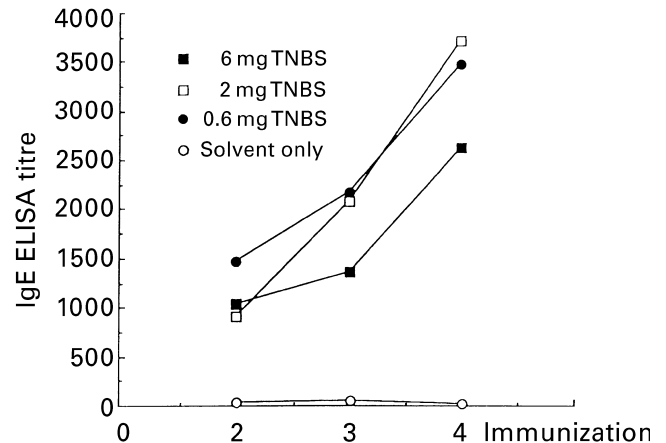


Fig. 3. The titres of TNP-specific IgE increase progressively after repeated immunization. BALB/c mice (15 mice per group) were repeatedly immunized with different doses of TNBS or solvent only by patch application in 3-week intervals. Sera were collected 1 week after each immunization. Titres of TNP-specific IgE were determined by ELISA individually. Dots represented the geometric means of each group.

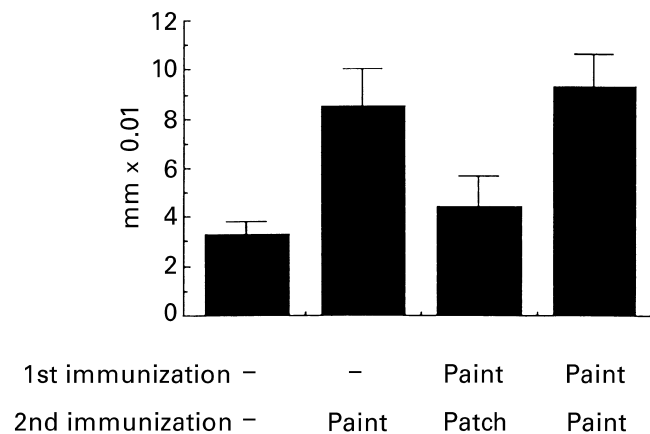


Fig. 4. Patch application-induced modulation is still effective on ever-immunized mice. BALB/c mice (seven per group) were all immunized with 6 mg TNBS through traditional painting first. Three weeks later, mice were reimmunized with 6 mg hapten through patch application or traditional painting. Five days after reimmunization, mice were challenged. The increased ear thickness was measured as in Fig. 2(c).

and challenged 5 days later as in the classic CHS model. The LNCs were obtained from donor mice immunized through patch application with hapten 5 days before, and were injected intravenously into recipients 1 h before sensitization or challenge. Mice receiving adoptive transfer before challenge showed reduced ear swelling, whereas those receiving adoptive transfer before sensitization had comparable ear swelling responses (Fig. 5). Similar adoptive transfer of immune LNCs obtained from donor mice, immunized through traditional painting 5 days before, showed no suppressive effect on either sensitization and elicitation phases of CHS (data not shown). Thus, the enhanced type 2 T-cell response induced by patch application has biological meaning which could negatively regulate CHS in the elicitation but not the sensitization phase.

CD4⁺ T cells are major effectors of the enhanced type 2 T-cell response induced by patch application

To determine the subsets of T cells mediating a type 2 response induced by patch application, an *in vitro* Ab blocking assay was performed. As shown in Fig. 6a, addition of anti-CD4 Ab, but not anti-CD8 Ab, almost completely inhibited the production of IL-4 in reactivation culture of LNCs from *in vivo*-primed mice. For further elucidation, the adoptive transfer system used in Fig. 5 was also conducted. The donor LNCs were first depleted of CD4⁺ or CD8⁺ T cells, respectively, by panning method before their transfer. Figure 6b shows that depletion of CD4⁺ T cells before transfer significantly reduced their suppressive effect, whereas CD8⁺ T cells played only a minor role. Collectively, these data indicated that the major effector cells of type 2 T-cell response induced by patch application are CD4⁺ T cells (TH2 cells).

Discussion

Antigen itself is a powerful controlling factor for type 1/type 2 differentiation. For example, the purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* preferentially activate TH1 or TH2 cells, respectively [23]. Hapten is a strong type 1 inducer in terms of its special bioactive character in conjugating proteins to form immunogens [24]. Recent breakthrough in understanding the molecular mechanism of hapten-T cell interaction may explain the reason. Haptens conjugate with many soluble or membrane-bound proteins or peptides with their chemically active motif [25]. The majority of T cells can recognize a hapten in association with different peptides [24,26]. Therefore, hapten-epitopes for 'sequence-independent' TCRs are comparatively more abundant and repetitive on a hapten-

modified cell surface than most 'conventional' peptide determinants. This may be the critical point for their strong type 1 T cell-inducing activity because a difference in the surface-density of a T-cell epitope has been proposed as a factor controlling type 1/type 2 differentiation [5,27]. Therefore, haptens administered through patch application, unlike protein antigens which induce a predominant TH2 response [20], induce an augmented TH2 response which cannot completely overcome the type 1 T-cell response.

The manner of administration can also influence immune deviation. This study took advantage of the device used in clinical patch test. Hence, antigens released from gauze entered the skin gradually and continuously. In contrast, acetone/oil solution of antigen was painted directly onto mice skin in the classic CHS model. Therefore, all hapten molecules, given their small size, penetrate skin very quickly [25]. It has been shown that continuous administration of protein antigen preferentially activates TH2 cells [28]. So, administration of antigen through patch application may promote TH2 responses. In addition, the dosage of antigen could modulate CD4⁺ T-helper phenotype development in murine and human culture system [29,30]. Bretscher *et al.* [31] and Bancroft, Else and Grencis [32] applied this strategy to animal models with parasite

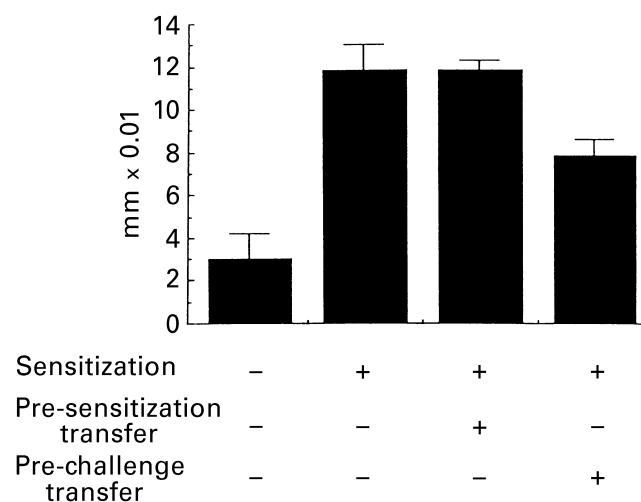


Fig. 5. Enhanced type 2 T-cell response can negatively regulate CHS in the elicitation phase, but not the sensitization phase. Recipient BALB/c mice (five per group) were sensitized with 6 mg TNCB by traditional painting and challenged with 1% TNCB 5 days later. They received adoptive cell transfer (4×10^7 immune LNCs/mouse) by i.v. injection 1 h before sensitization or challenge. The immune LNCs were obtained from donor BALB/c mice ($n = 12$) receiving immunization with 6 mg TNBS through patch application 5 days previously. The increase in ear thickness (mm $\times$ 0.01) was measured before and 24 h after challenge. The results were from one representative experiment out of three performed.

infection and showed that by manipulating the parasite doses, TH1/TH2 deviation could be tilted to such a degree that the susceptible mice could mount a protective immune response. In contrast with protein antigen, these data showed that the dose of hapten had no effect on type 1/type 2 balance of CHS.

Although the murine CHS model has been intensively investigated for several decades, the role of type 2 T-cell immune response in this animal model remains controversial [13–19]. Results here, which clearly showed that augmented hapten-specific type 2 T-cell immune responses could suppress the elicitation reaction, support the notion that type 2 T-cell immune responses play a negative regulatory role in the murine CHS model. The data also agree with Schwarz *et al.*'s [15] and Gautam, Chikkala and Hamilton's [17] reports which showed that IL-10 and IL-4 were ineffective in modulating the development of murine CHS when the cytokines were administered in the sensitization phase, but can reduce the CHS response when given in the elicitation phase. The reason why type 2 T-cell immune responses have different regulatory effects on these two responsive phases of murine CHS model remains obscure. The result here that CD4⁺ T cells are major effectors of type

2 T-cell response is consistent with previous reports. Gocinski and Tigelaar reported that *in vivo* depletion of CD4⁺ T cells resulted in strikingly enhanced ear swelling responses in mice [33]. Abe *et al.* further confirmed this phenomenon in a molecular approach [34]. They also demonstrated that type 2-cytokine-secreting, negative regulatory T cells express CD4 phenotype [8].

Type 1/Type 2 T-cell deviation has been proved to play an important role in the pathogenesis of many human diseases [3,4]. Allergic contact dermatitis has long been documented as type 1-predominant T-cell immune responses [6,7]. However, recently some authors have challenged this notion by demonstrating the presence of hapten-specific TH2 cells and expression of TH2 type cytokines in lesions of allergic contact dermatitis [10–12]. In addition, Kapsenberg *et al.* claimed that aberrant cytokine secretion could not determine the contact allergic state by showing similar TH1 type cytokine profiles of Ni-specific T-cell clones from Nickel contact allergic and nonallergic individuals [35]. However, in terms of the strong type 1-immune response-inducing character and the type 1/type 2 antagonizing phenomena shown here, it is proposed that contact allergic state is a type 1-predominant

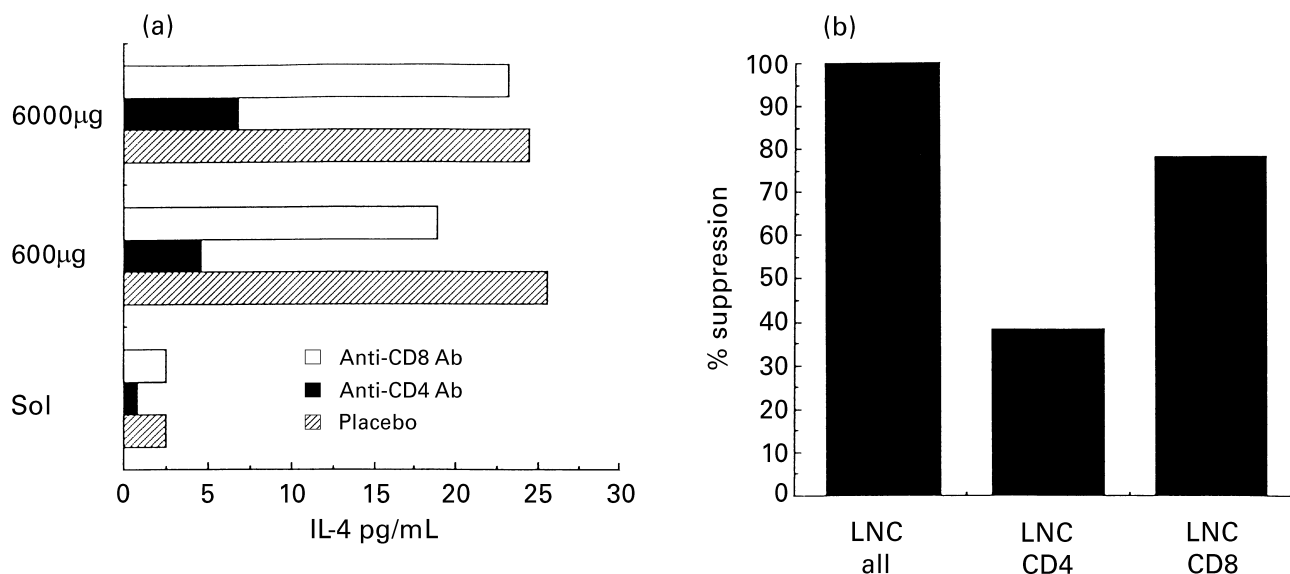


Fig. 6. The major mediating cells of enhanced type 2 T-cell response were CD4⁺ T cells. (a) BALB/c mice (three per group) were immunized with 6 mg, 0.6 mg TNBS or solvent only through patch application. One week later, draining lymph nodes were dissected. Pooled LNCs (2×10^6 /well) were co-cultured with irradiated, TNP-modified spleen cells (4×10^6 /well) from naive BALB/c mice in 96-well U-bottom plates alone or with addition of anti-CD4 Ab or anti-CD8 Ab in final concentration of $1 \mu\text{g/mL}$, respectively. Supernatants were collected at 48 h, and the amount of IL-4 secretion was measured with ELISA kit from Endogen. (b) Recipient BALB/c mice (five per group) were immunized with 6 mg TNBS by traditional painting and challenged 5 days later. The recipients received adoptive transfer of immune LNCs or CD4⁺ T cell-depleted LNCs or CD8⁺ T cell-depleted LNCs (4×10^7 /mouse) by i.v. injection 1 h before challenge, or left alone. The immune LNCs were obtained from donor BALB/c mice ($n = 22$) which received immunization with 6 mg TNBS through patch application 5 days ago. Portions of immune LNCs were predepleted of the CD4⁺ or CD8⁺ subset by the panning method. The suppression mediated by nondepleted LNCs was labelled as 100% and percentage of suppression mediated by CD4⁺ or CD8⁺ T cell-depleted LNCs was shown. The results were from one representative experiment out of three performed.

T-cell immune response and that the infiltrating TH2 cells may play a negative feedback regulatory role.

The skin, lung and gastrointestinal tract are the first lines of defence exposed to the external environment. Reports from this laboratory as well as from other researchers have demonstrated that IgE production after inhalation of protein antigen was transient and suppressed by Ag-specific $\gamma\delta$ T cells later [21,36]. Similar suppression was observed after oral administration of Ags [37]. However, in a previous report here [20], it was demonstrated that repeated epicutaneous exposures of protein antigen induced sustained, progressively increasing IgE levels. This phenomenon of sustained IgE production was observed again in the current study even though hapten is a very strong type 1 inducer. How the skin can persistently sustain the TH2 response is worth further clarify.

In conclusion, it was demonstrated that by modulating the manner of epicutaneous administration of hapten through patch application, type 1/type 2 T cell balance could be tilted so that the enhanced hapten-specific TH2 response could downregulate the type 1 T cell-predominant CHS. Further investigation of this strategy may be able to throw light on the regulatory mechanisms involved in contact allergy.

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