

Immunoglobulin E production in mice by means of contact sensitization with a simple chemical, hapten

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Production of IgE caused by repeated topical application of 2,4-dinitrofluorobenzene (DNFB) to the ears of BALB/c mice was investigated. Ear thickness increased in proportion to the number of applications. Eczematous changes of the skin included marked infiltration of neutrophils, eosinophils, and monocytes and hypertrophy of the epidermis. Ear thickness due to inflammation reached a maximum 24 hours after the second, third, fourth, and fifth applications. The strong expression of interferon- γ and IL-2 messenger RNA (mRNA) in the skin lesions indicated the participation of Th1 type helper T cells in the delayed-type hypersensitivity reaction. After the fifth application, the mice showed an immediate cutaneous reaction in addition to the delayed-type hypersensitivity reaction. The immediate reaction appeared within 1 hour of application. Hapten-specific IgE also was detected in serum from the mice, and the expression of germ-line and productive C ϵ mRNA was detected in cervical lymph nodes, whereas productive C ϵ mRNA was detected in the spleen. These results indicate that five topical applications of DNFB to the ear produce local eczematous dermatitis and increase serum hapten-specific IgE level in mice. Eczematous dermatitis is mainly caused by Th1 type T cells, and IgE production is mediated by Th2 type T cells in the cervical lymph nodes. In addition, IgE class switching occurs in the cervical nodes and IgE production occurs in both lymph nodes and spleen. (*J Allergy Clin Immunol* 1997;100:S39-44.)

Key words: Contact dermatitis, Th1 cell, Th2 cell, IgE, 2,4-dinitrofluorobenzene

Atopic dermatitis is characterized by severe eczematous dermatitis associated with high serum levels of IgE.¹⁻⁶ Atopic dermatitis often affects patients with a personal or family history of atopic eczema, allergic rhinitis, or atopic asthma, suggesting that IgE has an important role in the development of atopic dermatitis. Although the cause of atopic dermatitis is unknown, there is much evidence to suggest the participation of contact sensitization to allergens and food allergies. Some reports indicate production of IgE in atopic dermatitis after contact sensitization.⁷⁻¹¹ Saloga et al.⁷ reported that skin sensitization to ovalbumin induced production of specific IgE and IgG1 as well as an immediate cutaneous response. In addition to protein

Abbreviations used

CDNA:	Complementary deoxyribonucleic acid
DNFB:	2,4-dinitrofluorobenzene
DNP:	Dinitrophenyl
IFN:	interferon
mRNA:	messenger RNA
RNA:	ribonucleic acid
RT-PCR:	reverse-transcriptase polymerase chain reaction
T-PBS:	Phosphate-buffered saline solution containing 0.2% Tween 20

antigens, Dearman et al.⁸⁻¹⁰ reported that some simple chemical allergens, trimellitic anhydride and diphenylmethane-4,4'-diisocyanate, induced a delayed-type cutaneous hypersensitivity reaction and an increase in the serum concentration of hapten-specific IgE. They also reported that 2,4-dinitrochlorobenzene exhibited a clear potential for contact sensitization but apparently lacked the ability to induce an IgE response. Kitagaki et al.¹¹ reported that repeated cutaneous application of 2,4,6-trinitro-1-chlorobenzene induced a hapten-specific IgE response in mice. This study was conducted to examine the possibility of inducing an IgE response by means of repeated contact sensitization of mice with a simple chemical contact antigen, 2,4-dinitrofluorobenzene.

METHODS

Animals

Male BALB/c mice (7 to 10 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed in plastic cages in an air-conditioned room maintained at 24°C and were given a standard laboratory diet and free access to water. All experiments were performed according to the guidelines for the care and use of experimental animals proposed in 1987 by the Japanese Association for Laboratory Animal Science.

Sensitization with DNFB and measurement of ear thickness

The ears of the mice were painted with DNFB (Nacalai Tesque, Kyoto, Japan) or vehicle once a week for 5 weeks. Twenty-five microliters of 0.15% DNFB in a vehicle (acetone and olive oil [4:1]) was applied to each side of the ear. Ear thickness was measured with an engineering micrometer (R1-A; Ozaki MFG, Tokyo, Japan) and was expressed as the increase from time 0.

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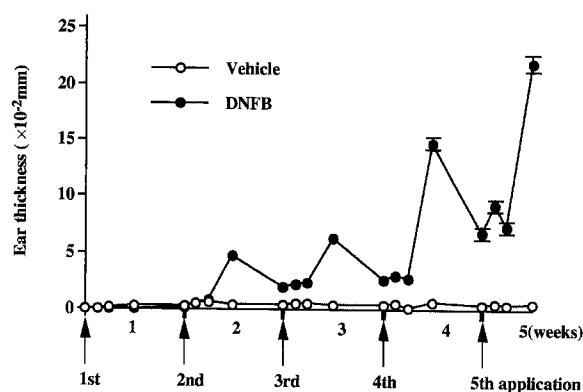


FIG. 1. Time course of changes in ear thickness. The thickness was assessed at 0, 1, 4, and 24 hours after each application of vehicle or DNFB. Data represent the arithmetical mean $\pm$ SEM for the indicated number of mice.

Histologic examination

Ears were removed from the mice 24 hours after the fifth application of the vehicle or DNFB and were fixed with 10% neutral formalin. The ears were cut into parasagittal sections, dehydrated, and embedded in paraffin by means of standard procedures. Paraffin sections were stained with hematoxylin and eosin for light microscopic examination.

Determination of serum antibody levels

The serum concentrations of hapten-specific IgE, total IgE, total IgG, and total IgM in the mice were measured by means of ELISA as described later. Serum samples were obtained from the mice 24 hours after the fifth application of DNFB.

We measured hapten-specific IgE titer with a captured ELISA.^{12, 13} In brief, immunoplates (EIAII Plus Microtitration plate; Flow Laboratories, McLean, Va.) were coated with rat monoclonal anti-mouse IgE (LO-ME-3, IgG1; Serotec, Oxford, UK) by means of incubation overnight at 4°C. The plates were blocked with phosphate-buffered saline containing 1% ELISA grade bovine serum albumin (Sigma, St. Louis, Mo.), incubated at room temperature for 1 hour, and washed three times with phosphate-buffered saline solution containing 0.2% Tween 20 (T-PBS). Mouse monoclonal anti-dinitrophenyl (DNP) IgE (SPE-7; Sigma) was sequentially diluted as the standard. A diluted serum sample (100 μ l) was added to each well, and the plates were incubated at room temperature for 1 hour. After washing with T-PBS, 100 μ l of diluted biotinylated DNP-bovine serum albumin was added to each well and incubated at room temperature for 1 hour. DNP-bovine serum albumin (LSL, Tokyo, Japan) was biotinylated with an N-hydroxysuccinimidyl-LC biotinylation kit (Pierce Chemical, Rockford, Illinois). After extensive washing with T-PBS, 100 μ l of dilute peroxidase-conjugated streptavidin (Dakopatts, Glostrup, Denmark) was added to each well. The reaction was stopped by means of addition of 100 μ l of stop solution after incubation at room temperature for 1 hour. The optical density of the reaction mixture was determined with an automatic ELISA plate reader (Titertek Multiscan MCC/340; Flow Laboratories) at 450 nm.

To measure total IgE, total IgG, and total IgM levels, immunoplates were coated by means of incubation overnight at 4°C with dilute rat monoclonal anti-IgE (LO-ME-3; Serotec), goat anti-mouse IgG (IgG; Organon Teknika, Turnhout, Belgium), and goat anti-mouse IgM (IgM; Organon Teknika),

respectively. The plates were blocked as described earlier and washed three times with T-PBS. Standard curves were generated as described earlier with mouse monoclonal anti-DNP IgE (SPE-7; Sigma), mouse IgG (Miles Scientific), and mouse IgM (Miles Scientific, Naperville, Ill.). One hundred microliters of serum was added to each well and incubated at room temperature for 1 hour. ELISA was performed with peroxidase-conjugated goat anti-mouse IgE (Nordic Immunological Laboratories, Tilburg, Netherlands), IgG (IgG; Organon Teknika), and IgM (IgM; Organon Teknika). The ELISA data for the samples were compared with the standards added to each plate by means of a Delta Soft program (ELISA Analysis; BioMetallics, Cupertino, Calif.) on a Macintosh computer. Titers of hapten-specific IgE and total IgE were expressed in ELISA units (EU)/ml on the basis of laboratory-generated standards. The total IgG and total IgM titers are given as μ g/ml based on appropriate commercial standards.

Analysis of cytokine mRNA expression in cervical lymph nodes and ear tissues

Changes in cytokine messenger ribonucleic acid (mRNA) levels were investigated in cervical lymph nodes and ear tissues by means of reverse transcriptase-polymerase chain reaction (RT-PCR) with a thermal cycler (Trio-Thermoblock; Bio-Mettra, Grottingen, Germany). Using Isogen (Nippon Gene, Tokyo, Japan), we prepared total RNA from the ears and lymph nodes of mice 4 hours after the fifth application of vehicle or DNFB. The total RNA level in each sample was measured by means of spectrophotometry at a wavelength of 260 nm, and the quality of the RNA was checked by means of electrophoresis. We performed RT-PCR as follows. Total RNA (500 ng) was reverse transcribed for 60 minutes at 42°C with a 1st-Stranded TM complementary deoxyribonucleic acid (cDNA) Synthesis kit (Clontech Lab, Palo Alto, Calif.). Samples of cDNA were amplified in a total volume of 100 μ l containing 0.5 μ mol/L of each primer (RT-PCR primers set; Stratagene) with a PCR reagent kit (GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase; Perkin Elmer, Branchburg, N. J.). The internal control was β -actin. The mixture was overlaid with mineral oil, and 30 and 35 PCR cycles were performed in a thermal cycler to detect β -actin, interferon (IFN)- γ , IL-2, IL-4, and IL-5. The PCR products were resolved by means of electrophoresis and stained with ethidium bromide to reveal DNA. Samples were obtained from three mice, and the ears and cervical lymph nodes were removed 24 hours after the fifth application of DNFB.

Analysis of germline and productive C ϵ gene expression

An RT-PCR was performed as described earlier to assess changes in cervical lymph node- and spleen-derived germline and productive C ϵ mRNA levels. RNA (500 ng) was reverse-transcribed for 60 minutes at 42°C with a 1st-Stranded TM cDNA synthesis kit. The cDNA samples were amplified in a total volume of 100 μ l containing 0.5 μ mol/L of each 5'- and 3'-primer (for germline C ϵ : 5'-primer I ϵ , ACTAGAGATTCA-CAACG; 3'-primer C ϵ 2, AGCGATGAATGGAGTAGC; sequences for productive C ϵ : 5'-primer j4, TGGACTACT-GGGGTCAAGG; 3'-primer C ϵ 2, AGCGATGAATGGAGTAGC)¹⁴ with the GeneAmp PCR reagents.

The PCR involved 30 cycles in a thermal cycler of denaturation at 94°C for 5 minutes and annealing at 60°C for 5 minutes followed by 30 cycles of 1.5 minutes at 72°C, 1.5 minutes at 94°C, and 1.5 minutes at 60°C with final extension

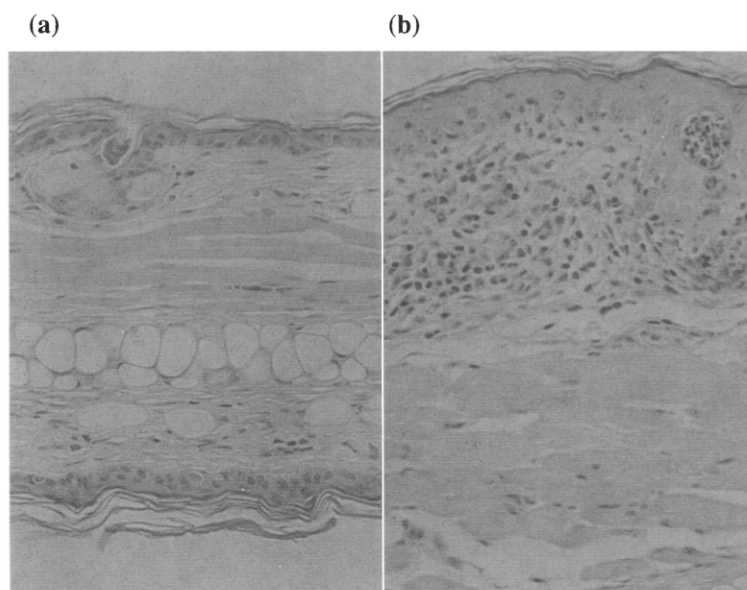


FIG. 2. Photomicrographs of skin lesions 24 hours after the fifth application of vehicle (A) or DNFB (B) (hematoxylin and eosin stain; original magnification $\times 200$).

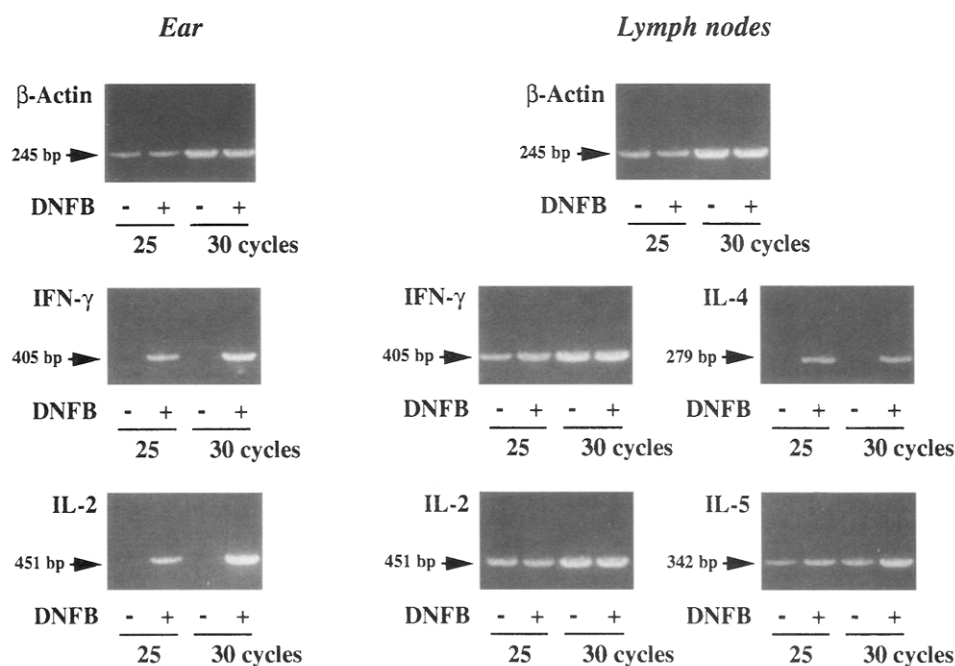


FIG. 3. Cytokine mRNA expression in the ears and lymph nodes 4 hours after the fifth application of vehicle or DNFB. *bp*, base pair.

for 10 minutes at 72° C. The internal control was β -actin. PCR products were resolved by means of electrophoresis and visualized with ethidium bromide. Cervical lymph nodes and spleens were obtained 48 hours after application of DNFB.

Measurement of CD40 expression

The cervical lymph nodes were removed and lymphocyte suspensions were prepared to study CD40-positive B cells 24

hours after the fifth application of DNFB. Lymph node cells were stained with a fluorescein isothiocyanate-labeled hamster monoclonal anti-CD40 (IgM; PharMingen, San Diego, Calif.). After staining with propidium iodide, CD40-positive cells were counted with a fluorescence-activated cell sorter (FACSscan II, Becton Dickinson, San Jose, Calif.) and the LYSIS II program (Becton Dickinson). Propidium iodide-positive cells were gated out as dead cells.

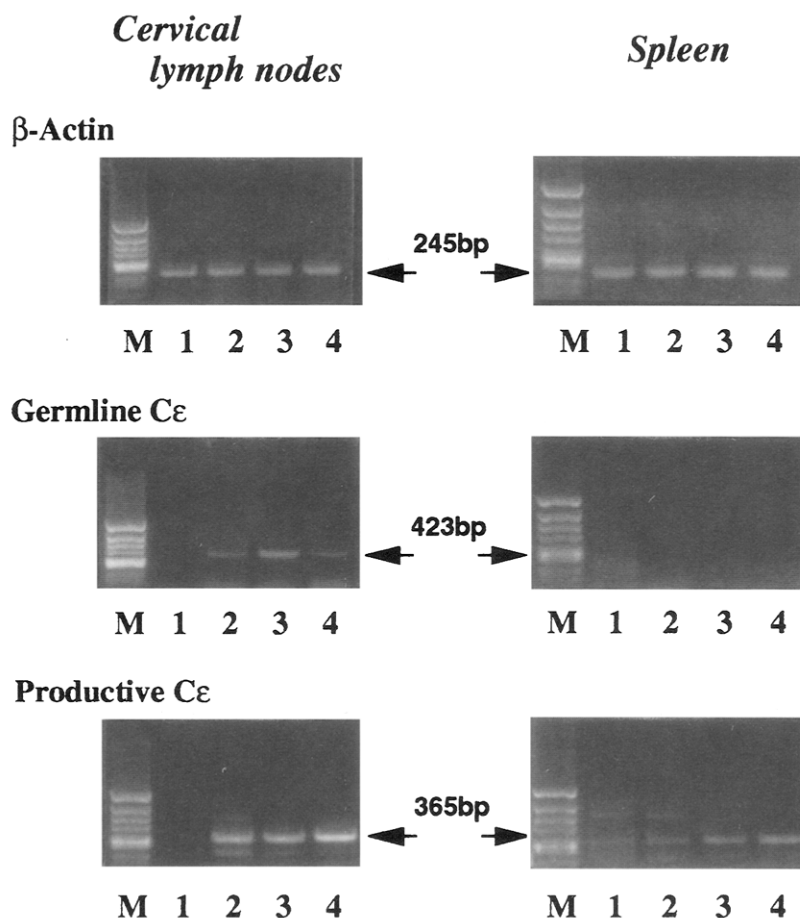


FIG. 4. Expression of germline and productive C ϵ mRNA in the cervical lymph nodes and the spleen. Expression of mRNA was examined 48 hours after DNFB application. Lane M, size marker; lane 1, day 2; lane 2, day 9; lane 3, day 23; lane 4, day 30. *bp*, base pairs.

Analysis of lymphocytes in cervical lymph nodes

Cervical lymph node cells were obtained from mice sensitized with five applications of DNFB. A single-cell suspension (1×10^6 cells) was incubated for 30 minutes at 4°C with fluorescein isothiocyanate-conjugated monoclonal anti-Thy1.2 (5a-8, mouse IgG2b; Caltag Laboratories, San Francisco, Calif.) and phycoerythrin-conjugated monoclonal anti-B220 (RA3-6B2, rat IgG2a; Caltag) to determine the T cell-to-B cell ratio, with fluorescein isothiocyanate-conjugated monoclonal anti-CD8 (53-6.7, rat IgG2; PharMingen) and phycoerythrin-conjugated monoclonal anti-CD4 (RM4-5, rat IgG2a; PharMingen) for assessment of the CD4/CD8 cell ratio and with fluorescein isothiocyanate-conjugated monoclonal anti-CD4 (RM4-5, rat IgG2a; PharMingen) and phycoerythrin-conjugated monoclonal anti-CD25 (3C7, rat IgG2b; PharMingen) for measurement of CD25/CD4 cell ratio. The cells were washed twice with phosphate-buffered saline solution and then stained with propidium iodide.

Multiparameter flow cytometry was performed with a FACScan II cell sorter, and the data were processed with the LYSIS II program. Lymphocyte populations were gated by means of forward scatter and side scatter parameters, and propidium iodide-positive cells were gated out as dead cells.

RESULTS

Changes in the ears

Repeated topical application of DNFB to the ears provoked typical contact dermatitis on the mice. Fig. 1 shows the time course of the change in ear thickness, which increased in proportion to the number of applications of DNFB. Baseline ear thickness was $21.68 \pm 0.18 \times 10^{-2}$ mm. Contact dermatitis was detected 24 hours after the second, third, fourth, and fifth applications of DNFB. With the fifth application, an immediate reaction was seen 1 hour after painting in addition to the 24-hour reaction. Fig. 2 shows photomicrographs of the ear skin 24 hours after the fifth application of either vehicle or DNFB. Marked infiltration of inflammatory cells (such as neutrophils, eosinophils, and monocytes) and hypertrophy of the epidermis were observed among the DNFB group. No marked changes were observed among the vehicle group. Cytokine mRNA expression in ear tissue 4 hours after the fifth application of DNFB was analyzed by means of the RT-PCR method (Fig. 3). A high level of expression of IFN- γ and IL-2 mRNA was

TABLE I. Effect of repeated application of DNFB on serum antibody levels

Antibody titer	sIgE (EU/ml)	tIgE (EU/ml)	tIgG (μ g/ml)	tIgM (μ g/ml)
Week 0 ($n = 7$)	0	365 $\pm$ 76	117 $\pm$ 25	563 $\pm$ 78
Week 5, vehicle ($n = 18$)	0	173 $\pm$ 43	1456 $\pm$ 61	745 $\pm$ 70
Week 5, DNFB ($n = 19$)	163 $\pm$ 25	4909 $\pm$ 433	1235 $\pm$ 48	571 $\pm$ 42

The titers of hapten-specific IgE (sIgE), total IgE (tIgE), total IgG (tIgG) and total IgM (tIgM) in mouse serum were measured during week 0 and during week 5 4 hours after the fifth application of vehicle or DNFB. Readings for sIgE and tIgE are given in ELISA units (EU)/ml relative to laboratory-generated DNP-sIgE and tIgE standards. Values for tIgG and tIgM are given in μ g/ml relative to respective commercial standards. Data represent the arithmetical mean $\pm$ SEM for the indicated number of mice.

detected in the ears of DNFB-treated mice but not of the vehicle-treated mice. However, mRNA for IL-4 or IL-5 was not detected in either group of mice (data not shown).

Serum IgE level

A statistically significant increase in hapten-specific IgE and total IgE levels was observed among mice treated five times with DNFB. There was no significant difference in total IgG and IgM levels between the vehicle and DNFB groups (Table I).

Changes in cervical lymph nodes and spleen

The effect of repeated application of DNFB on the weight of the cervical lymph nodes and spleen was evaluated. The cervical lymph nodes in the DNFB group (10.3 ± 0.5 mg, $n = 4$) were about threefold heavier than those of the vehicle group (3.0 ± 0.4 mg, $n = 4$), but the spleen weight was similar in both groups (vehicle, 97.6 ± 10.5 mg, $n = 4$; DNFB, 116.4 ± 7.2 mg, $n = 4$). Lymphocyte populations in the cervical nodes were analyzed by means of flow cytometry 4 hours after the fifth application of vehicle or DNFB. The ratio of T to B cells (Thy1.2⁺/B220⁺ ratio) showed a decrease for the DNFB group, but there was no difference in CD4⁺/CD8⁺ ratio or CD25⁺/CD4⁺ ratio between the two groups (Table II).

Evaluation of cytokine mRNA expression in the cervical lymph nodes showed that IFN- γ and IL-2 mRNA were always present. Among the mice given five applications of DNFB, strong expression of IL-4 and IL-5 mRNAs also was observed (Fig. 3). Whereas expression of productive C ϵ mRNA was detected in both the cervical lymph nodes and the spleen, expression of germline C ϵ mRNA was observed only in the lymph nodes (Fig. 4). When the number of CD40-positive cells in the cervical lymph nodes of mice given five applications of DNFB or vehicle, the number of CD40-positive

TABLE II. Effect of repeated application of DNFB on lymphocyte populations in the cervical lymph nodes

Ratio	Thy1.2 ⁺ /B220 ⁺ (T/B)	CD4 ⁺ /CD8 ⁺	CD25 ⁺ /CD4 ⁺
Vehicle	2.39 $\pm$ 0.0970	2.40 $\pm$ 0.0968	0.153 $\pm$ 0.0127
DNFB	1.25 $\pm$ 0.0835	2.44 $\pm$ 0.0827	0.162 $\pm$ 0.0185

Mice were killed 4 hours after the fifth application of vehicle or DNFB. Cervical lymph node cells from individual mice were examined by means of flow cytometry. Data represent the arithmetical mean $\pm$ SEM for six to nine mice.

cells increased in the DNFB group. The number of CD40-positive cells increased in a way among the DNFB group.

DISCUSSION

In this study, we demonstrated that repeated application of DNFB to the skin induced topical contact dermatitis and an increase in hapten-specific IgE. This hapten-induced contact dermatitis was mainly due to delayed hypersensitivity mediated by Th1 type T cells,^{15,16} as confirmed with the strong expression of IFN- γ and IL-2 mRNA in the skin lesions of the mice. Five applications of DNFB also induced production of serum hapten-specific IgE. This was confirmed with the appearance of an immediate skin reaction 1 hour after the fifth application. In addition, there was a high level of expression of IL-4 and IL-5 mRNA in the cervical lymph nodes, corresponding to the raised serum IgE level, although expression of this mRNA was not detected in the skin lesions. This suggested that IgE production was evoked by Th2 type T cells in the cervical lymph nodes but not in the skin lesions. The proliferation of B cells in the cervical lymph nodes (shown with the expression of CD40) is additional evidence. DNFB is generally considered to be a potential contact allergen that can cause contact dermatitis. However, our data indicate that repeated application of DNFB also resulted in a specific IgE response.

Dearman et al.^{8,9} reported that only some specific allergens that cause both contact sensitization and respiratory allergy, such as trimellitic anhydride and 2,4-dinitrochlorobenzene, are able to invoke an IgE response. In contrast to these agents, DNFB does not appear to induce respiratory hypersensitivity. In this study, the mice did not show any respiratory abnormalities after application of DNFB, even though immediate cutaneous hypersensitivity was detected. Kitagaki et al.¹¹ also reported that repeated application of 2,4,6-trinitro-1-chlorobenzene resulted in an IgE response and an immediate cutaneous response, but they did not detect any respiratory changes after application of the antigen. These findings suggest that a contact allergen, which is only capable of causing contact allergy, can also invoke an IgE response and an immediate skin reaction without affecting the respiratory system.