

## Original Article

# A third-phase cutaneous (very late phase) response after elicitation with dinitrofluorobenzene in passively or actively sensitized mice

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### ABSTRACT

Previous studies have reported that the mice passively sensitized with anti-dinitrophenol (DNP) IgE antibody exhibited IgE-mediated cutaneous reaction with an immediate phase response (IPR) at 1 h and a late phase response (LPR) at 24 h after the challenge of dinitrofluorobenzene (DNFB). We found that the third-phase inflammatory reaction with intense and persisting infiltration of eosinophils, named 'very late phase reaction (vLPR)', was induced following IPR and LPR in response to DNFB in actively and passively sensitized mice, and that the peak response of vLPR was at 8 days after the challenge. This reaction was slightly observed in non-sensitized mice. Since the accumulation of eosinophils in vLPR was markedly observed when compared with that of LPR at 24 h, the vLPR may be an important reaction in allergic diseases. The development of vLPR was partly decreased in mast cell-deficient WBB6F1-W/W<sup>v</sup> mice and was absent in T cell-deficient BALB/c-nu/nu mice in passive sensitization. These results indicate that the vLPR in the triphasic cutaneous reaction may be mainly mediated by T cells and partially by mast cells and/or IgE antibody, and consequently lead to an intense ear swelling accompanying massive infiltration of eosinophils.

**Key words:** anti-dinitrophenol IgE antibody, dinitrofluorobenzene, eosinophilic infiltration, late phase response, very late phase response.

### INTRODUCTION

Recent studies have shown that allergen-induced reaction in the skin and airway consists of two inflammatory reactions: an immediate phase response (IPR) and a late phase response (LPR).<sup>1–6</sup> Immediate phase response IPR is primarily caused within 1 h after the antigen exposure by IgE-dependent activation of mast cells, resulting in release of pro-inflammatory mediators,<sup>5</sup> and is often followed by an intense inflammatory reaction termed LPR.<sup>1</sup> Late phase response appears 3–48 h after the elicitation and is considered to be an important inflammatory reaction due to its similarity to the clinical manifestation of chronic allergic diseases and its difficulty of suppression by antiallergic drugs without side effects. The increase of mast cells is essentially observed in IPR, while LPR is characterized by the accumulation of inflammatory cells including neutrophils, mononuclear cells and eosinophils.<sup>5,6</sup>

We have shown that spikelets of *Miscanthus sinensis* and some Kampo medicines inhibit the biphasic skin reaction induced by intravenous injection of monoclonal anti-dinitrophenol (DNP) IgE antibody in mice and subsequent skin testing with dinitrofluorobenzene (DNFB).<sup>7–9</sup> In the process of our study, we recently found a third inflammatory-phase response following LPR, temporarily named 'very late phase reaction (vLPR)'. In the present study, we investigated the characteristics of vLPR and the histopathologic features in mice passively sensitized with anti-DNP IgE antibody.

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Received 14 January 1999. Accepted for publication 10 June 1999.

## METHODS

### Mice

Specific pathogen-free BALB/c mice (6-week-old), BALB/c-nu/nu mice (6-week-old), WBB6F1- +/+ mice (8-week-old) and WBB6F1-W/W<sup>v</sup> mice (8-week-old) were purchased from Japan SLC Inc., Hamamatsu, Japan, and maintained in the Laboratory for Animal Experiments, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, Japan. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University, Japan.

### Antigens and chemicals

Dinitrofluorobenzene was purchased from Nacalai Tesque, Kyoto, Japan, and dissolved in 100% ethanol. Dinitrophenol-derivation of ovalbumin (DNP-OVA) was performed by the method of Eisen *et al.*<sup>10</sup> The DNP-OVA preparation was calculated to contain 3.5 DNP groups per OVA molecule. Aluminum hydroxide gel (alum) was prepared according to the method of Levine and Vaz<sup>11</sup> and used as an adjuvant for the immunization with DNP-OVA antigen. Bovine Serum Albumin (BSA) was purchased from Sigma, St Louis, MO, USA, and dissolved in saline before use.

### Anti-DNP IgE preparation

An anti-DNP monoclonal antibody (mAb)-producing cell line (EC1) was cultured in 10 mL of an equal volume mixture of RPMI-1640 and Dulbecco's modified Eagle's medium, with high glucose supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Life Technologies, Inc., Grand Island, NY, USA) and 2 mmol/L glutamine until reaching confluence. The supernatant was harvested, centrifuged at 400 g and stored at -80°C until used.<sup>12</sup> The IgE antibody titer was estimated to be 1:1024 by heterologous passive cutaneous anaphylaxis in rats injected intravenously with DNP-bovine serum albumin as antigen.<sup>13</sup>

### Induction of skin reaction in mouse ears

BALB/c mice were actively or passively sensitized with DNP-OVA or anti-DNP IgE mAb, respectively. In the passive sensitization model, mice were given an i.v. injection of a 1 mL aliquot of anti-DNP IgE mAb-containing

fluid 24 h before the DNFB challenge. Skin reaction was elicited by applying 10 µL of 0.1%, 0.01% or 0.001% DNFB in 100% ethanol to each side of each ear of sensitized mice. The reaction to DNFB was evaluated by measuring ear thickness using a dial thickness gauge (G-1 A type, Peacock; Ozaki MFG, Co., Ltd, Osaka, Japan) immediately before the challenge and at appropriate intervals after. The results were expressed as average ear swelling (increase in ear thickness, µm) ± SD of three to five mice. For active sensitization, mice were immunized intraperitoneally (i.p.) with 10 µg DNP-OVA admixed with 1 mg of alum 2 weeks before the DNFB challenge. The mice were injected in each ear with 25 µL of OVA (1 µg/µL) and BSA (1 µg/µL) solution. All other procedures were the same as for passive sensitization. At the time of DNFB challenge, serum IgE antibody titer against DNP was estimated to be 1:512 by heterologous passive cutaneous anaphylaxis (hPCA).

### Histologic examinations

The control and treated mice were killed under anesthetized conditions at appropriate times after DNFB challenge, and the ears removed. The tissues were fixed with 4% paraformaldehyde solution and embedded in paraffin after dehydration with a series of ethanol. The paraffin sections were stained with hematoxylin and eosin, toluidine blue, or naphthol AS-D chloroacetate (esterase). The corresponding cells were counted under a light microscope in five sections of 5 mm length at a magnification ×1000.

### Statistical analysis

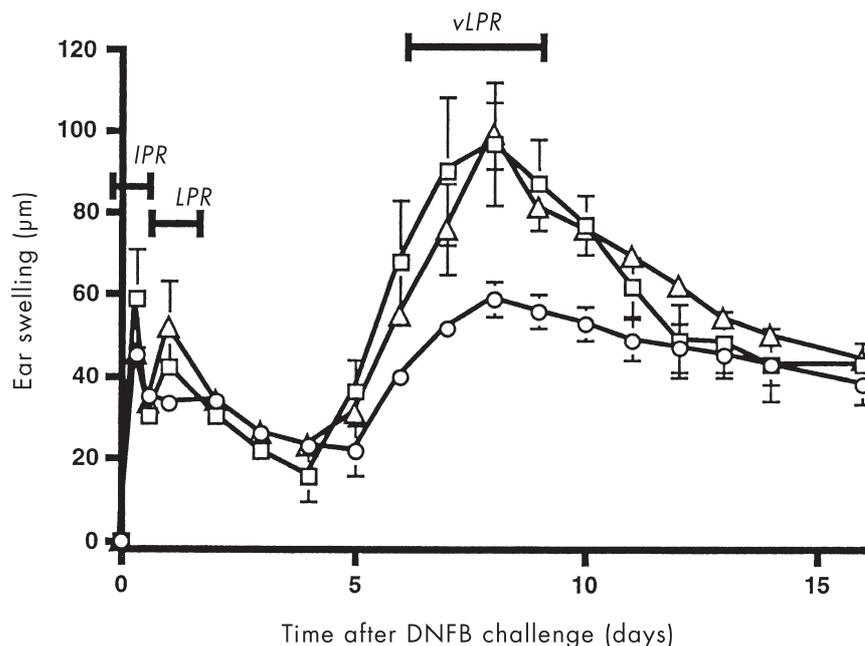
Statistical significance of difference between the groups was determined by the Mann-Whitney's *U*-test (ear swelling experiment) or Student's *t*-test (histologic examination).

## RESULTS

### Time course of IgE-mediated skin reaction in passively or actively sensitized mice

Figure 1 shows the time course of IgE-mediated cutaneous reaction in mice that were passively and actively sensitized with anti-DNP IgE antibody and DNP-OVA plus alum, respectively. In passively sensitized mice, biphasic skin reaction consisting of IPR and LPR was induced within 3 days after the challenge of DNFB. The peak response of IPR was at 1 h, and LPR response at 24 h after the skin test. These results were well consistent with the previous

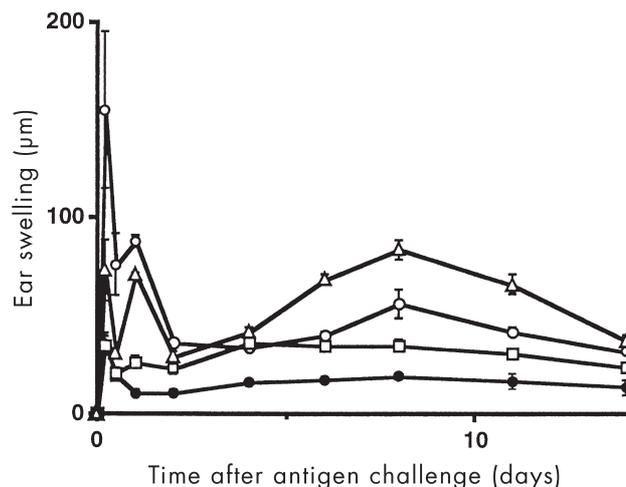
**Fig. 1** Time course study of dinitrofluorobenzene (DNFB)-specific skin reaction in mice. BALB/c mice were actively or passively sensitized with 10  $\mu$ g dinitrophenol (DNP)-ovalbumin (OVA) and 1 mg alum or 1.0 mL anti-DNP IgE monoclonal antibody (mAb) preparation, 2 weeks or 24 h before antigen challenge, respectively. Skin reaction was elicited by applying 0.1% DNFB in 100% ethanol to the ear skin of the actively ( $\Delta$ ) and passively ( $\square$ ) sensitized and non-sensitized ( $\circ$ ) mice. Each value represents mean ear swelling ( $\mu$ m)  $\pm$  SD of three mice. IPR, immediate phase response; LPR, late phase response; vLPR, very late phase response.



findings.<sup>7</sup> Interestingly, a third intense cutaneous reaction (ear swelling) following LPR was observed during 5–10 days after the DNFB challenge, peaking at approximately 8 days. After 1 month, the ear swelling gradually decreased to a normal level. The third-phase reaction was temporarily designated 'very late phase reaction (vLPR)'. The degree of vLPR was more intense and sustained than LPR after the skin test. Very late phase reaction was also observed after the challenge of DNFB in actively sensitized mice, with a similar pattern to that in passively sensitized mice. In contrast, LPR was not induced in non-sensitized mice, and the ear swelling at 8 days after the challenge was much less than that of the passively and actively sensitized mice. Thus, the presence of a third-phase cutaneous reaction (i.e., vLPR) following IPR and LPR in response to DNFB in sensitized mice was clearly established.

### Applying OVA instead of DNFB in actively sensitized mice

To clarify the effect of DNFB, triphasic skin reaction was elicited by applying OVA. In active sensitization, mice were immunized by DNP-OVA and alum and were injected with 25  $\mu$ L OVA or BSA or saline into each ear 14 days after sensitization. Dinitrofluorobenzene was painted in the same way as for passive sensitization. Triphasic skin reaction was observed in the OVA-injected ear, but vLPR disappeared in the BSA-injected ear. (Fig. 2)



**Fig. 2** Time course of triphasic skin reaction in actively sensitized mice. Mice were sensitized by an intraperitoneal injection with 10  $\mu$ g dinitrophenol (DNP)-ovalbumin (OVA) and 1 mg alum. Two weeks later they were injected with 25  $\mu$ L saline ( $\bullet$ ), 25  $\mu$ g OVA ( $\circ$ ) or 25  $\mu$ g BSA ( $\square$ ) or painted with 20  $\mu$ L dinitrofluorobenzene ( $\Delta$ ) on their ear. Each value represents mean  $\pm$  SD of three mice.

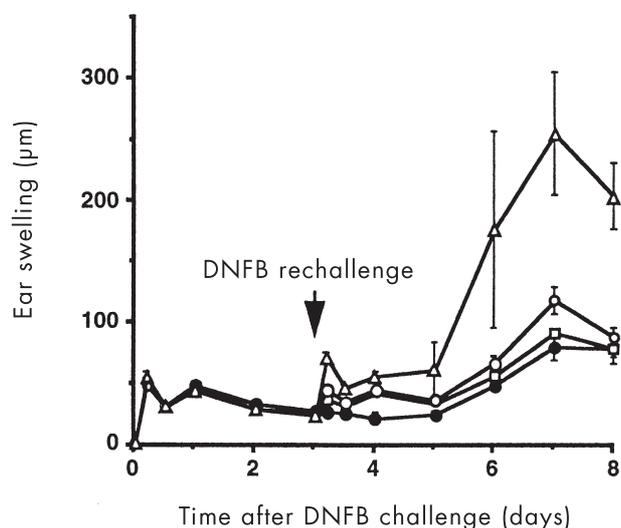
### Re-challenge of DNFB on IgE-mediated cutaneous skin reaction

The above findings indicate that triphasic skin reactions (IPR, LPR and vLPR) were induced by DNFB challenge in passively or actively sensitized BALB/c mice. It might be

supposed that vLPR was a result of a re-challenge of DNFB from the paws of mice, acquired from wiping and scratching during the process of the biphasic skin reaction. Therefore, to confirm this, we investigated the influence of the DNFB re-challenge in passively sensitized mice. Skin reaction was elicited by applying 0.1% DNFB to each ear of sensitized mice. Immediate phase response and LPR were confirmed at 1 h and 24 h after the challenge. On day 3, various concentrations (0.1%, 0.01% and 0.001%) of DNFB were re-challenged to the ear skin. As shown in Fig. 3, IPR and LPR were observed at 1 h and 24 h after the second challenge of DNFB in a dose-dependent manner. The increased peak response of vLPR appeared at 7 days after the first DNFB challenge, dose dependently. These results strongly suggest that the development of vLPR was not due to the secondary exposure of DNFB.

### Histopathologic study of skin reaction

Figure 4 shows that the number of esterase-positive cells (neutrophils, and macrophages) slightly increased in a close relation to the skin reaction (ear swelling) at 1 h and 24 h, but that there was no discernible change in the number of toluidine blue-positive cells (mast cells) after

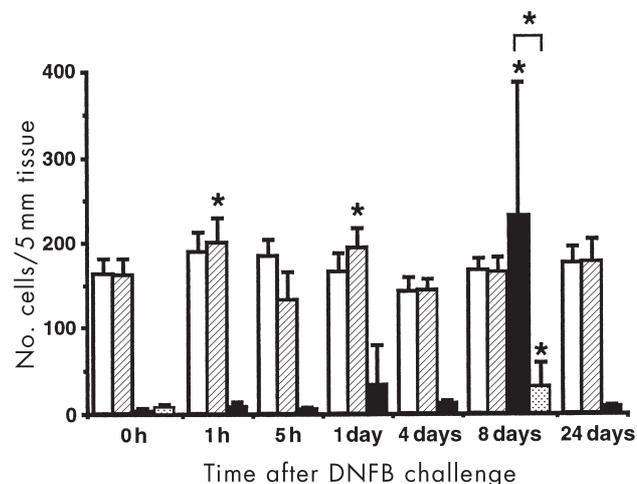


**Fig. 3** Influence of dinitrofluorobenzene (DNFB) re-challenge on IgE-mediated skin reaction in passively sensitized mice. Mice were passively sensitized with anti-dinitrophenol (DNP) IgE mAb 24 h before skin testing with 0.1% DNFB in 100% ethanol. On day 3 after the first challenge, mice were re-challenged with 0.001% (□), 0.01% (○), 0.1% DNFB (△) or ethanol alone (●) for skin testing. Each value represents mean ear swelling (µm) ± SD of three mice.

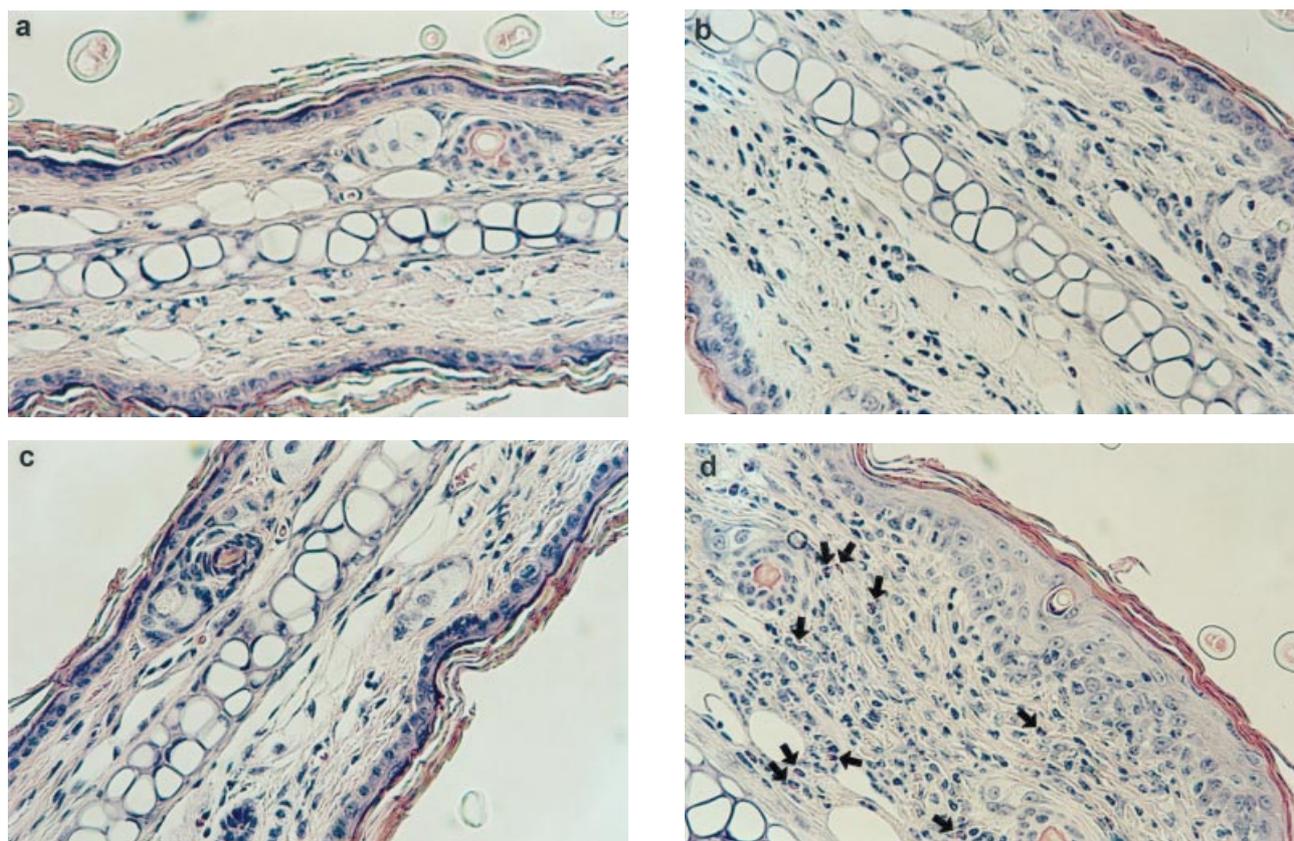
the challenge. Eosinophils were rarely seen in the ear of passively sensitized mice before the DNFB challenge, and conspicuously increased at 24 h (LPR) after the challenge. Although they then decreased at 4 days, a massive infiltration into the tested ear skin was then observed at 8 days after the challenge. In non-sensitized mice, the number of eosinophils increased at 8 days, but was much less than that of passively sensitized mice.

Figure 5 shows the histopathology of the ear of BALB/c mice challenged with DNFB, following staining with hematoxylin and eosin. One day after the challenge, infiltration of inflammatory cells was observed with edema of the dermis (Fig. 5b) when compared with skin before the challenge (Fig. 5a). No significant change was seen at 4 days after the DNFB challenge (Fig. 5c), but eosinophils then infiltrated in conjunction with vLPR into the challenged ear, particularly around hair roots (Fig. 5d). Epidermal proliferation was also observed in some lesions of ears of passively sensitized mice at 8 days.

It is therefore of major interest that the marked epidermal proliferation was observed in vLPR at 8 days after



**Fig. 4** Histopathologic analysis of skin reaction in passively sensitized mice. Mice received intravenous injection of 1.0 mL anti-dinitrophenol (DNP) IgE mAb preparation 24 h before skin testing with 0.1% dinitrofluorobenzene (DNFB) in 100% ethanol. After DNFB challenge, the mice were killed and the ears were removed for histopathologic examination. The thin sections were stained with toluidine blue, naphthol-AS-D-chloroacetate esterase or hematoxylin and eosin. The toluidine blue-positive (□), esterase-positive (▨), eosinophilic cells (▤) in passively sensitized mice and eosinophilic cells in non-sensitized mice at 0 and 8 days (■) were manually counted under a light microscope in five sections of 5 mm length. Each value represents mean ± SD of five sections. \* $P < 0.05$  using the Student's *t*-test.



**Fig. 5** Histopathology of skin reaction in passively sensitized mice. Mice received intravenous injection of 1.0 mL anti-dinitrophenol (DNP) IgE mAb preparation 24 h before skin testing with 0.1% dinitrofluorobenzene (DNFB) in 100% ethanol. After DNFB challenge, the mice were killed and the ears were removed for histopathologic examination. Thin sections were stained with hematoxylin and eosin. (a) Skin of mouse ear before DNFB challenge. (b) At 24 h after challenge; moderate edema of the dermis and inflammatory cell infiltrations can be seen. (c) At 4 days after challenge; no significant changes compared to normal skin can be seen. (d) At 8 days after the challenge; marked infiltration of eosinophils (arrows) and epidermal proliferation are observed.

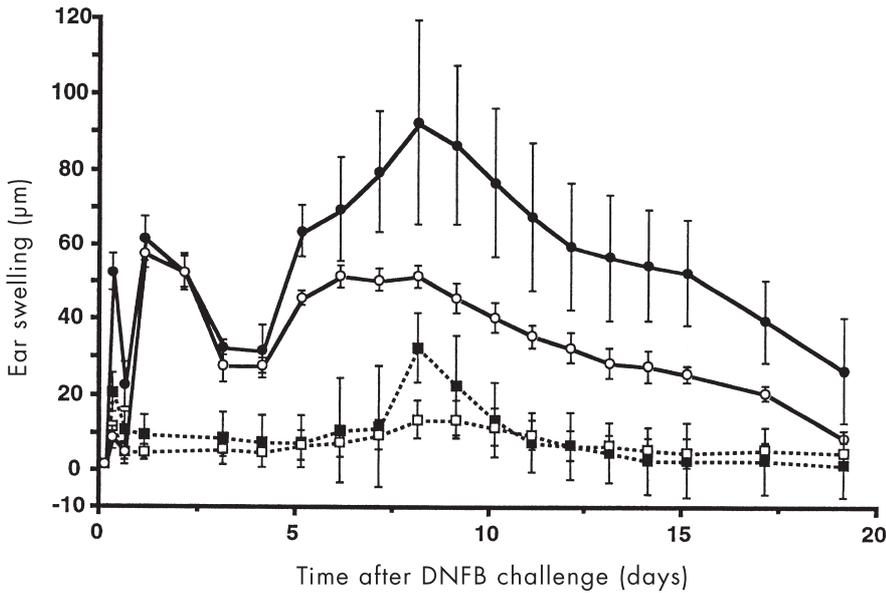
the challenge. Previous studies indicated that the infiltration of mast cells may play an essential role in IPR, and that LPR may be primarily elicited by the participation of other cells, including macrophages and/or neutrophils.<sup>7</sup> The present study revealed that eosinophils are prominently involved in the development of vLPR.

#### Elicitation of IgE-mediated skin reaction in mast cell- or T cell-deficient mice

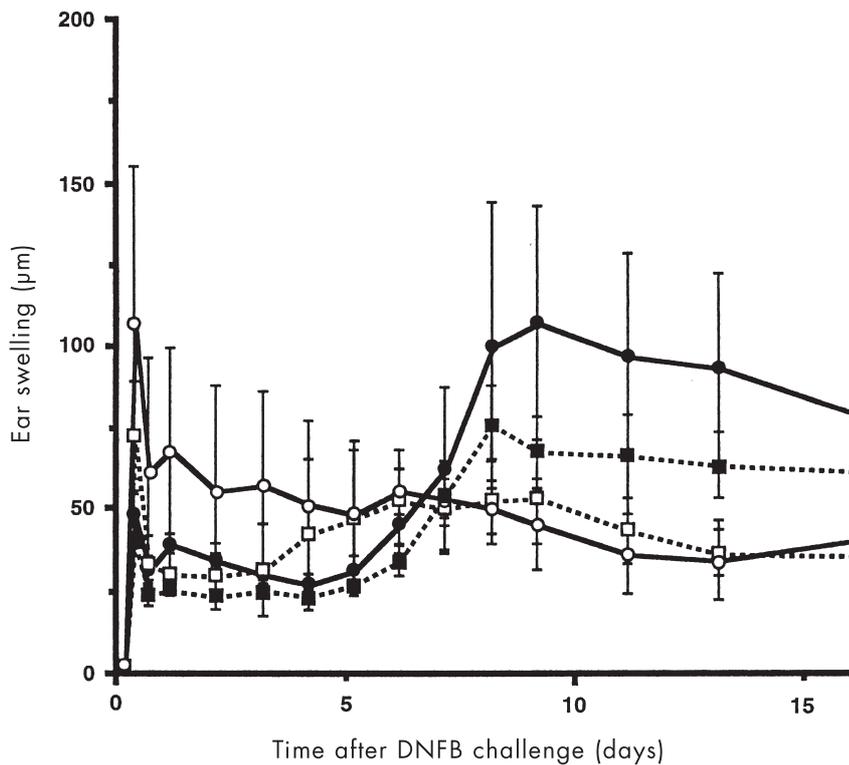
To examine the participation of mast cells and T cells in vLPR following epicutaneous challenge, we investigated the triphasic cutaneous reaction in genetically deficient mice that were passively sensitized with anti-DNP IgE antibody. Immediate phase response was absent in mast

cell-deficient WBB6F1-W/W<sup>y</sup> mice when compared with their congenital litter mates W/W +/+, but LPR was sufficiently observed (Fig. 6). On the other hand, vLPR was apparently attenuated in WBB6F1-W/W<sup>y</sup> mice. In non-sensitized W/W +/+ mice, diminished vLPR was elicited when compared with passively sensitized W/W +/+ mice. But vLPR was not observed in non-sensitized W/W<sup>y</sup> mice.

As shown in Fig. 7, both IPR and LPR were surveyed after the DNFB challenge in passively sensitized BALB/c nu/nu mice, and the degree of the response was greater than in BALB/c mice. In contrast, vLPR had essentially disappeared in BALB/c nu/nu mice, but was strongly present in BALB/c mice. In non-sensitized BALB/c nu/nu mice, a slight increase of ear swelling was detected, conforming with vLPR.



**Fig. 6** Time course study of IgE-mediated skin reaction in passively sensitized WBB6F1 mice. Mast cell-deficient WBB6F1-W/W<sup>-</sup> mice (○) and their litter mates W/W +/+ (●) were passively sensitized with 1.0 mL anti-dinitrophenol IgE mAb preparation 24 h before skin testing with 0.1% dinitrofluorobenzene (DNFB) in 100% ethanol and WBB6F1-W/W<sup>-</sup> (□) and W/W +/+ (■) mice were non-sensitized. Each value represents mean ear swelling (µm) ± SD of three mice.



**Fig. 7** Time course study of IgE-mediated skin reaction in passively sensitized BALB/c-nu/nu mice. T cell-deficient BALB/c-nu/nu mice (○) and their litter mates (●) were passively sensitized with 1.0 mL anti-DNP IgE mAb preparation 24 h before skin testing with 0.1% dinitrofluorobenzene (DNFB) in 100% ethanol and BALB/c-nu/nu (□) and BALB/c (■) mice were non-sensitized. Each value represents mean ± SD of three mice.

**DISCUSSION**

Several investigations have reported that mice passively sensitized with IgE-containing solution exhibited immediate and late phase skin reactions to the subsequent challenge of antigen.<sup>1,2,5,6</sup> The inflammation associated with LPR is of great clinical importance as it accounts for

the morbidity and severity of chronic allergic diseases like bronchial asthma, rhinitis and atopic dermatitis.<sup>14</sup> Late phase response has been shown to accompany polymorphic inflammatory infiltrates such as neutrophils, eosinophils, and lymphocytes.<sup>5,6</sup> Particularly, eosinophils are responsible for this phenomenon in IgE-mediated skin reaction as important effector cells.<sup>2,5,6,15</sup> However,

many studies have reported that LPR in IgE-mediated skin reaction was observed at most 72 h after the antigen exposure.<sup>5-8,15-17</sup>

In the present study, we noticed that passive sensitization with anti-DNP IgE antibody followed by the challenge of DNFB to mouse ears can induce the triphasic cutaneous reactions of IPR, LPR and vLPR, peaking at 1 h, 24 h and 8 days after antigen challenge, respectively (Fig. 1). The third-phase inflammatory response was more intense for ear swelling than LPR, and persisted for longer periods. Very late phase response was markedly induced in actively sensitized mice as well as passively sensitized mice, but was only slightly observed in non-sensitized mice. The triphasic cutaneous reactions appeared with injection of OVA in actively sensitized mice, which indicates that vLPR may be due to an antigen (hapten)-specific response in sensitized mice, but not specific for DNFB.

The peak response of vLPR was observed at 7 or 8 days after the challenge of DNFB, even with a re-challenge on day 3 after the first challenge (i.e., following IPR and LPR; Fig. 3). This indicates that the induction of vLPR may not depend on the re-contact with DNFB, but may result from internal factors or their related cells produced by the first challenge.

Histopathologic examination revealed massive infiltration of eosinophils in vLPR at 8 days, suggesting that eosinophils are responsible for the development of this reaction. However, no marked increase of eosinophils in peripheral blood was observed at the time of vLPR in passively sensitized mice (data not shown). Although many studies have shown that eosinophilic infiltration was observed in LPR at 24 h after skin test,<sup>5,6,16</sup> our present results indicate that the accumulation of eosinophils at vLPR was more marked than at LPR in passively sensitized mice. This suggests that vLPR with eosinophil infiltration actually represents an important inflammatory reaction in allergic diseases. The LPR seen in bronchial disease<sup>18-21</sup> may be very similar to the vLPR in our study. Especially, Hutson *et al.* 1988<sup>20</sup> have reported two delayed bronchoconstrictor events, including a peak response at 17 h after challenge and a further response at 72 h with increased eosinophils in BAL from guinea-pig in an asthma model. Also, vLPR is apparently different from post late phase reaction (pLPR), meaning non-allergic hyperactivity in bronchial asthma,<sup>22</sup> because the third inflammation continued for very long periods and more intensely than LPR.

In genetically mast cell-deficient WBB6F1-W/W<sup>v</sup> mice

with mutation of the W/c-kit locus, IPR was absent but LPR was strongly manifested after DNFB challenge (Fig. 6). Very late phase response was also present, but to a lesser degree than in WBB6F1- +/+ mice. This finding indicates that mast cells or mediators originating from them may be a prerequisite for the development of vLPR. Because mutations in the W/c-kit locus result in the absence of the c-kit receptor or the production of abnormal receptors,<sup>23-25</sup> stem-cell factor, a ligand for the c-kit receptor, may be partly associated with the development of allergic response. Diminished vLPR was detected in non-sensitized W/W +/+ mice than in sensitized W/W +/+ mice, but disappeared in non-sensitized W/W<sup>v</sup> mice. These findings suggest that the presence of IgE antibody might enhance the development of vLPR. On the other hand, both IPR and LPR were induced after DNFB challenge in T cell-deficient BALB/c-nu/nu mice passively sensitized with anti-DNP IgE antibody, but vLPR was almost completely absent, in contrast to BALB/c mice. These results clearly indicate that LPR is a T cell-independent response, while vLPR is mainly mediated by T cells and factors derived from them. Because T cells and eosinophils have been reported to accumulate in the skin of a patient with atopic dermatitis,<sup>26-28</sup> further study will be needed to examine the close association between T cells and eosinophils in the skin reaction. A slight increase of ear swelling in non-sensitized mice was evident during 6-9 days, which might have been dependent on mast cell or its mediators released by the irritant chemical effect of DNFB. Allergic reaction has been considered to be divided into two separate categories: IgE-mediated response and delayed type hypersensitivity (DTH). Very late phase response might be a delayed type hypersensitivity (DTH), which is so-called 'flare-up' in the dermatology field, and weal and flare phenomenon observed at the late phase of the patch test. If vLPR were DTH, it might be considered to be DTH-enhanced by the presence of IgE. Therefore, in vLPR two allergic categories might be observed at the same time.

In the present study, we demonstrated for the first time that third-phase inflammatory response with massive eosinophilic infiltration, designated vLPR, was induced by the DNFB challenge in passively and actively sensitized mice. Very late phase response in the triphasic cutaneous reaction was mainly mediated by T cells, partially dependent on mast cells and/or IgE antibody, and obviously different from LPR. Although it has not been clarified what kinds of chemical mediators are involved in the formation of vLPR, our preliminary study indicates

that the expression of granulocyte-macrophage colony stimulating factor (GM-CSF) and regulated upon activation, normal T cell expressed and secreted (RANTES) mRNA in mouse ears was detected at vLPR by the reverse transcriptase polymerase chain reaction method. To understand the pathologic significance of vLPR in this triphasic cutaneous reaction, further studies will be required to clarify the mechanism underlying the development of vLPR, especially the marked infiltration of eosinophils and the pathologic relation between T cells and eosinophils.

## ACKNOWLEDGEMENTS

We thank Mr Y Kurashige and Ms K Hayashi for their technical assistance. This work is supported in part by the grant for the research of Oriental Medicine from Tokyo Metropolitan Government.

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