

The expression of murine cutaneous late phase reaction requires both IgE antibodies and CD4 T cells

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Summary

Background Exposure of atopic patients to a specific allergen evokes an immediate response which is followed, in many cases, by a late phase reaction (LPR) some hours later. Here we have examined the immunological mechanisms required for the expression of cutaneous LPR in mice.

Methods BALB/c mice were immunized by i.p. injection of ovalbumin (OVA) and alum actively or by i.v. injection of anti-OVA IgE monoclonal antibody (mAb) passively. After challenge by intradermal injection of OVA into ears, the changes in ear thickness, the number of eosinophils, and the levels of IL-4 and IFN- γ protein at the site of antigen challenge were examined.

Results Actively immunized mice developed a biphasic response at the site of OVA injection, while mice passively immunized with IgE anti-OVA mAb displayed a strong early response but no LPR. Cell transfer experiments using BALB/c nu/nu mice revealed that both OVA-specific IgE mAb and OVA-primed CD4 T cells were required to evoke LPR. Moreover, LPR was associated with increased levels of IL-4 production concomitant with reduced IFN- γ production and was abolished by pretreatment with anti-IL-4 neutralizing mAb.

Conclusion It is suggested that murine cutaneous LPR against OVA is a type 2 inflammatory response in which both IgE antibodies and CD4 T cells play an obligatory role.

Keywords: IgE, CD4 T cells, IL-4, anti-IL-4, IFN- γ , eosinophils, mast cells, mouse, late-phase reaction, ovalbumin

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Introduction

When allergic patients are challenged with an allergen against which they are sensitized, they rapidly develop an inflammatory reaction of short duration. This early response is caused primarily by IgE-dependent activation of mast cells/basophils resulting in the release of proinflammatory mediators. The early phase response is often followed after several hours by a delayed, more sustained local inflammation, termed late phase reaction (LPR) [1,2]. LPR is

characterized by the accumulation of activated inflammatory cells including eosinophils, monocytes and T lymphocytes [3]. The inflammation associated with LPR is of great clinical importance in that it accounts for the morbidity and severity of chronic allergic diseases like bronchial asthma, rhinitis or atopic dermatitis [4]. The mechanism leading to the development and maintenance of LPR, however, has not yet been fully elucidated.

According to some investigators, LPR is primarily an IgE- and mast-cell-dependent reaction [2,5], whereas according to others LPR mainly corresponds to a CD4 T cell-mediated inflammatory response [6]. The early clinical observations that mast cell inhibitors like cromolyn sodium

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prevent not only the early but also the late phase response to allergen challenge, strongly supported the role of mast cells in the induction of LPR [7]. However, more recent studies have underlined the proinflammatory activities of activated CD4 T cells infiltrating the site of chronic allergic inflammation. These CD4 T cells were shown to display a Th2 phenotype in that they express IL-4 and IL-5 but not IFN- γ as revealed by *in situ* hybridization [8,9] or by analysis of the T cell clones derived from biopsy specimens of lesional skin or bronchial mucosa [10].

That allergen-specific Th2 CD4 T cells play a causative role in atopic diseases was further suggested by the ability of these cells not only to promote IgE antibody synthesis [11] but also to recruit and activate inflammatory cells, including eosinophils, at the site of the allergic response [6]. This proinflammatory activity of Th2 cells is mediated by their liberation of IL-5, IL-4 and IL-3. However, it is important to note that mast cells/basophils may also release the same cytokines upon IgE-dependent activation [12]. The respective role of IgE/mast cells versus Th2 lymphocytes in LPR is therefore still unclear.

In the present study we have used a mouse model to examine the relative contribution of IgE antibodies and CD4 T cells to the development of the early and late phase response to the protein antigen (Ag). Animals immunized actively with ovalbumin (OVA) displayed a typical biphasic response upon intradermal injection of OVA; animals passively immunized with IgE mAb to OVA showed only a strong immediate response and those passively immunized with primed T cells did not respond. However, mice passively immunized with both IgE monoclonal antibody (mAb) and primed CD4 T cells mounted a biphasic response comparable to that of actively immunized mice, indicating that both IgE Ab and T cells are required to induce LPR against a protein Ag.

Materials and methods

Animals

Five- to 9-week-old male BALB/c mice (Clea Japan Inc., Fuji, Japan) and 6- to 9-week-old male BALB/c nu/nu mice (Charles River Japan Inc., Atsugi, Japan) were used.

Reagents

OVA was purchased from Sigma Chemical Co., St Louis, USA. Mouse anti-OVA IgE mAb was produced by fusing OVA-primed spleen cells of BALB/c mice with P3X63AGU.1 (ATCC, Bethesda, MD), according to the method of Köhler and Milstein [13]. Hybridomas secreting anti-OVA IgE mAb were selected based on the ability of

culture supernatants to sensitize rat skin in 48-h passive cutaneous anaphylaxis (PCA). Their specificity was confirmed in rat PCA, and resulting anti-OVA IgE (κ chain) mAb (TOE2) reacted with OVA and DNP-OVA, but not with human serum albumin or bovine serum albumin. Hybridoma cells were grown in serum-free Hymedium 606 (Kojin Bio Co. Ltd, Sakado, Japan) for 1 day at the concentration of 1×10^7 cells/ml, and culture supernatants (PCA titre: 1/512 or 1/1024) were used for passive immunization. Rat anti-mouse IL-4 mAb (11B11) and rat anti-mouse IFN- γ mAb (R4-6A2) were purchased from ATCC. For *in vivo* use, these two antibodies were prepared as ascitic fluid in BALB/c nu/nu mice, and then purified by passage on a protein A column (Bio-Rad, Richmond, CA). Normal rat IgG was purchased from Zymed Lab. Inc., San Francisco, USA.

Immunization

Mice were immunized by i.p. injection of 1 μ g OVA and 1 mg aluminium hydroxide gel (alum). Fourteen days after immunization, the serum levels of anti-OVA IgE Ab and anti-OVA IgG1 Ab were 243 ± 23 ng/ml and 1.67 ± 0.62 μ g/ml, respectively (mean $\pm$ SE of eight mice, representative of more than five experiments).

Measurement of skin reaction

Fourteen days after immunization with OVA and alum, or 2 days after i.v. injection with 0.2 or 0.5 ml of anti-OVA IgE mAb, mice were challenged with intradermal injection of 10 μ l of saline containing 10 μ g of OVA, to both ears. The ear thickness was measured using an engineer's dial thickness gage ($\times 10^{-2}$ mm), before and after challenge. In each experiment, age-matched control mice were challenged with the same amount of OVA or saline, and ear thickness was measured similarly to immunized mice. The average of the background increase in ear thickness in the control group at each point was subtracted from the increase in ear thickness in immunized mice, and the results were expressed as the ear swelling.

Histological examination

In some experiments, ears were excised at various times after Ag challenge. Samples were fixed with 10% formaline, and then stained with Luna's staining. Each preparation was counted for total number of eosinophils in full cross section under a microscope.

Cell transfer experiments

Fourteen days after immunization with OVA and alum, mice were killed and their spleens were removed. Single

cell suspensions were prepared by gentle teasing. In some experiments, spleen cells were depleted of CD4 T cells and CD8 T cells, by magnetic cell sorting with the use of L3T4 microbeads[®] and Ly2 microbeads[®] (Miltenyi Biotec Inc., Sunnyvale, California, USA), respectively; residual CD4 and CD8 T cells in each fraction were 1%. Alternatively, spleen cells were enriched for CD4 T cells by using Collectplus Mouse CD4 kit[®] (Biotex Laboratory Inc., Edmonton, Canada), and the purity of CD4 T cells was 90%. Unfractionated or fractionated spleen cells (2×10^7) were injected i.v. to BALB/c nu/nu mice that had been injected i.v. with saline or anti-OVA IgE mAb 2 days before testing. Recipients were challenged with OVA, 1 h after cell transfer.

Extraction of tissue IL-4 and IFN- γ

IL-4 and IFN- γ in ear tissue were extracted according to the method described by Ferguson *et al.* [14]. Briefly, at various times after Ag challenge, both ears were excised, weighed, and then added to 1.5-ml microcentrifuge tubes containing 0.5 ml of 0.1% Tween 20 in PBS. Samples were ground using a pellet pestle (Kontes, Vineland, New Jersey); they were frozen in liquid nitrogen, thawed in a water bath, again ground for 3 min on ice, and then sonicated for 15 s. Finally, they were centrifuged at $\times 15\,000$ rpm for 20 min. Supernatants were removed and kept at -80°C until ELISA assay.

IL-4 and IFN- γ measurement

Murine IL-4 was measured by sandwich ELISA with the use of rat anti-mouse IL-4 mAbs, BVD4-1D11 and biotinylated BVD6-24G2 (Endogen Inc., Boston, MA), as previously described [15]. The sensitivity of IL-4 assay was 10 pg/ml, and the assay did not react with murine IL-1 α , IL-1 β , IL-2, IL-3, IL-5, IL-6, IL-10, IFN- γ , GM-CSF or TNF- α . Murine IFN- γ was measured by sandwich ELISA using rat anti-mouse IFN- γ mAbs, R4-6A2 and biotinylated XMG1.2 (Pharmingen, San Diego, CA); the minimum sensitivity of the assay was 0.1 ng/ml, and the assay did not react with murine IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF or TNF- α .

Results

IgE Ab-mediated early response is not sufficient to induce cutaneous LPR

In the first series of experiments, we examined whether IgE Ab-dependent immediate response to Ag is followed by LPR. To this end, we measured the changes in ear thickness after intradermal Ag challenge of immunized and un-immunized mice. As shown in Fig. 1(a), mice passively immunized by i.v. injection of anti-OVA IgE mAb developed an immediate

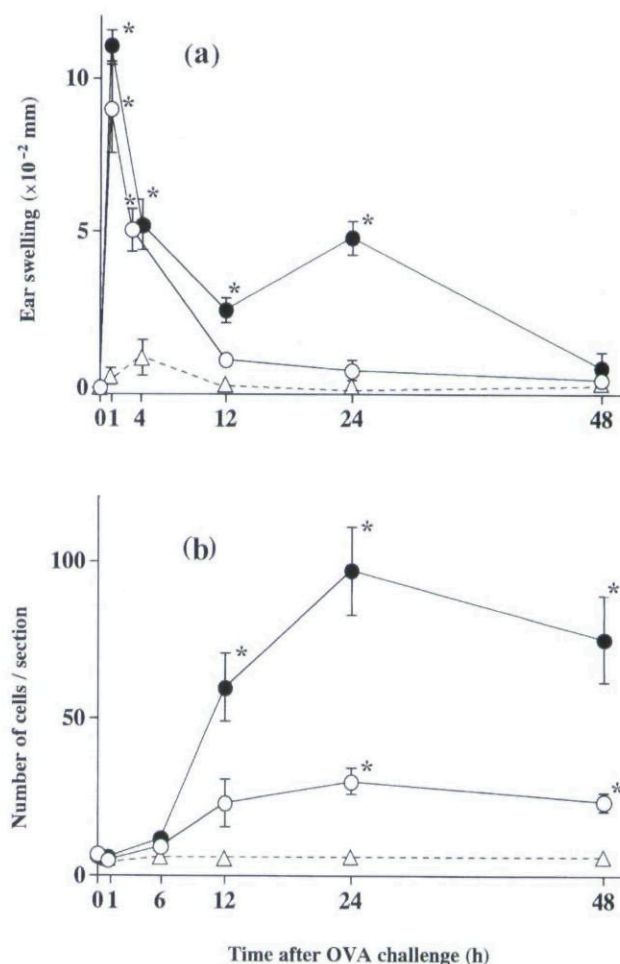


Fig. 1. Cutaneous response of actively or passively immunized mice. BALB/c mice were immunized actively by i.p. injection of 1 μg of OVA and alum 14 days previously (●), or passively by i.v. injection of anti-OVA IgE mAb 2 days previously (○). Immunized and unimmunized mice (△) were challenged by intradermal injection of 10 μg OVA into ears. (a) At the indicated times after challenge, ear thickness was measured and the background increase in ear thickness in unimmunized mice challenged with saline was subtracted. (b) Ears were excised and subjected to histological examination for counting the number of eosinophils. Results are expressed as the mean and SE of six animals. *: significant difference from unimmunized mice at $P < 0.01$ by Bonferroni's test.

response significantly higher than un-immunized mice after Ag challenge, while they did not develop LPR. In contrast, mice that were immunized 14 days prior to testing, by i.p. injection of OVA, developed a biphasic response that peaked at 1 and 24 h after Ag challenge. Similarly, DNP-OVA challenge induced a biphasic response in mice actively immunized against DNP-OVA but not in those passively immunized with anti-DNP IgE mAb (data not shown).

Table 1. Requirements for the induction of biphasic ear response in BALB/c nu/nu mice

	Passive immunization	Transferred cells		n	Ear swelling ($\times 10^{-2}$ mm)	
		Donor	Fraction		1 h	20 h
Exp. 1	No	—	Saline	6	0.87 ± 0.24	0.17 ± 0.18
	Yes	—	Saline	6	4.78 ± 0.22	-0.04 ± 0.58
	No	Immunized	Unfrac.	6	0.87 ± 0.30	-0.12 ± 0.30
	Yes	Immunized	Unfrac.	6	4.37 ± 0.47	1.63 ± 0.31
	Yes	Immunized	CD4-positive	6	4.62 ± 0.51	2.54 ± 0.35
Exp. 2	Yes	Immunized	Unfrac.	6	5.50 ± 0.65	1.83 ± 0.47
	Yes	Immunized	CD4-negative	4	6.00 ± 0.74	-0.13 ± 0.43
	Yes	Immunized	CD8-negative	5	6.40 ± 1.22	1.85 ± 0.24
Exp. 3	Yes	—	Saline	4	2.83 ± 0.55	-0.05 ± 0.60
	Yes	Normal	Unfrac.	4	2.70 ± 1.06	-0.17 ± 0.24
	Yes	Immunized	Unfrac.	4	2.70 ± 0.46	1.58 ± 0.24

BALB/c nu/nu mice were passively immunized with anti-OVA IgE mAb 2 days before cell transfer. Unfractionated spleen cells (unfrac.) were obtained from normal or OVA-immunized BALB/c mice. Cells were enriched for CD4 T cells or were depleted of CD4 T cells or CD8 T cells. Fractionated or unfractionated cells were transferred i.v. to recipients. Recipients were challenged with OVA as indicated in Fig. 1, and ear thickness was measured at 1 and 20 h. The background increase in ear thickness was determined in unimmunized BALB/c nu/nu mice challenged with saline (Exp. 1) or OVA (Exp. 2 and Exp. 3). Each value indicates the mean and SE. Significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) by Student's test (Exp. 1 and Exp. 3) or by Bonferroni's test (Exp. 2).

Further, we examined the accumulation of eosinophils at the site of Ag challenge in actively or passively immunized mice. As shown in Fig. 1(b), Ag challenge of OVA-immunized mice caused a local accumulation of eosinophils which peaked at 24 h, while this cellular infiltration in mice immunized with anti-OVA IgE mAb was significantly lower than OVA-immunized mice. These results strongly suggest that IgE antibody itself is not sufficient to promote the development of LPR.

Both IgE antibody and Ag-primed CD4 T cells are required for the induction of LPR

To determine whether IgE Ab-dependent early response is required for the development of LPR, cell transfer experiments, in combination with passive immunization with anti-OVA IgE mAb, were carried out. The results are summarized in Table 1. BALB/c nu/nu mice that received i.v. injection of spleen cells prepared from OVA-immunized

Table 2. Effect of OVA challenge on the amount of IL-4 and IFN- γ protein in ear skins of BALB/c mice

Immunization	n	IL-4 (ng/g tissue)		IFN- γ (ng/g tissue)	
		Before	After	Before	After
—	9	0.65 ± 0.10 (0.29–1.12)	0.83 ± 0.07 (0.53–1.15)	0.52 ± 0.08 (0.00–0.92)	0.44 ± 0.03 (0.30–0.58)
OVA	15	0.59 ± 0.07 (0.16–1.20)	$2.55 \pm 0.51^*$ (0.68–8.55)	0.74 ± 0.16 (0.00–2.37)	0.68 ± 0.26 (0.00–2.89)
Anti-OVA IgE	14	0.61 ± 0.07 (0.28–1.16)	0.52 ± 0.17 (0.12–2.12)	0.40 ± 0.03 (0.25–0.70)	0.47 ± 0.13 (0.00–1.24)

BALB/c mice were immunized actively with OVA and alum or passively with anti-OVA IgE mAb as indicated in Fig. 1. Ears were excised before or 24 h after OVA challenge. They were ground, and supernatants were determined for IL-4 and IFN- γ by ELISA. Each value indicates the mean and SE with minimum and maximum value in parenthesis.

*: $P < 0.01$, significant difference from the value before Ag challenge by Aspin-Welch's *t*-test.

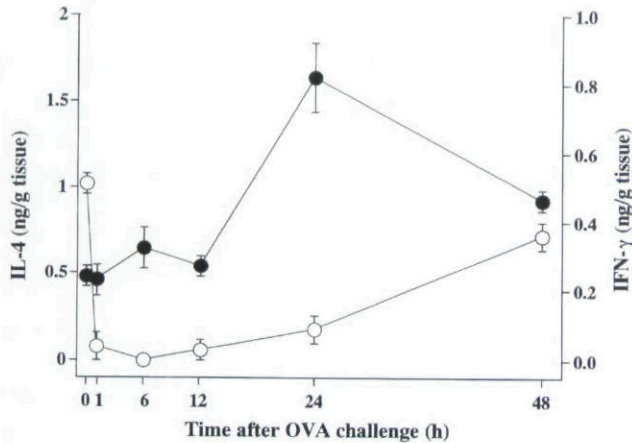


Fig. 2. Changes in the amount of IL-4 and IFN- γ protein in ears of OVA-immunized mice. Representative of four experiments. BALB/c mice were immunized by i.p. injection of OVA and alum 14 days before testing. They were challenged with OVA as described in Fig. 1. Ears were excised at the indicated times after Ag challenge. The amounts of tissue IL-4 (●) and IFN- γ (○) were determined by ELISA. Results are expressed as the mean and SE (ng/g tissue) of six mice.

mice developed an immediate response and LPR after Ag challenge if they had been immunized passively with anti-OVA IgE mAb, but they did not develop LPR if they had not been immunized with anti-OVA IgE mAb. Moreover, CD4 T cells enriched from spleen cells of OVA-immunized mice have a capacity to support the development of LPR. In addition, OVA-primed spleen cells depleted of CD4 T cells failed to support the development of LPR, while OVA-primed spleen cells depleted of CD8 T cells retained the ability to support it.

Taken together with the observation that normal spleen cells did not support the development of LPR in recipients even if they had been immunized passively with anti-OVA IgE mAb, these results suggest that IgE Ab-dependent early response is a prerequisite for the induction of a local immune response in which Ag-primed CD4 T cells play a crucial role.

Detection of IL-4 and IFN- γ protein at the site of Ag challenge

To characterize the cytokine phenotype of the local immune response that is involved in LPR, we measured IL-4 and IFN- γ protein in ear skin of mice, before or 24 h after Ag challenge, by using specific ELISA. Table 2 shows the representative results. Low levels of IL-4 and IFN- γ were detected in the ears of normal mice; these cytokine levels were not affected by Ag challenge of either normal or passively immunized mice. In contrast, a several-fold

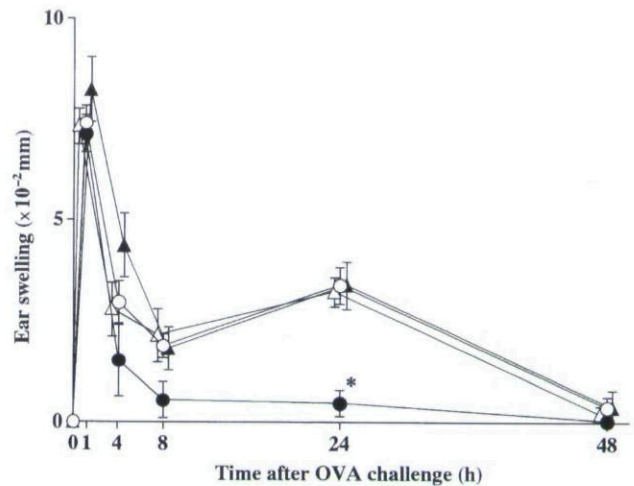


Fig. 3. Pretreatment with anti-IL-4 mAb prevents the development of LPR in OVA-immunized mice. Representative of two experiments. BALB/c mice were immunized actively by i.p. injection of OVA and alum. Fourteen days later, animals received i.v. injection with saline (○), 0.5 mg of control rat IgG (△), anti-IL-4 mAb (●), or anti-IFN- γ mAb (▲). Unimmunized mice were challenged with OVA, and the background increase in ear swelling was measured. Results are expressed as the mean and SE of six animals. *: significant difference from saline-injected control group at $P < 0.01$ by Dunnett's test.

increase in the amount of IL-4, but not of IFN- γ , was observed after Ag challenge of OVA-immunized mice. As shown in Fig. 2, IL-4 reached a maximum level 24 h after Ag challenge and returned to the baseline level at 48 h. In contrast, IFN- γ decreased at 1 h, remained low for 12 h, and returned to the baseline level at 48 h. These results strongly suggest that LPR is associated with Th2, but not Th1, immune response.

Effect of anti-IL-4 mAb on Ag-induced biphasic ear response

Further to the report that Th2 cell-induced tissue inflammation is blocked by neutralization of IL-4 [16], we examined the effect of anti-IL-4 mAb on a biphasic ear swelling in OVA-immunized mice. As shown in Fig. 3, anti-IL-4 mAb treatment abolished the development of LPR, without affecting the early response. In contrast, control rat IgG or anti-IFN- γ mAb had no influence on the early or late phase responses.

Discussion

Understanding the mechanisms leading to LPR is of major clinical relevance given the close association between the intensity of airway mucosa or skin chronic inflammation

and the severity of bronchial asthma or atopic dermatitis. Two mechanisms have been proposed to account for the development of LPR in allergic individuals. The first underlines the role of IgE and mast cells/basophils [2,5] and the other emphasizes the contribution of allergen-specific type 2 CD4 T cells [4,8,9]. Using a mouse model of early- and late-phase allergic cutaneous reaction to soluble protein Ag, we here provide compelling evidence that both IgE Ab and type 2 CD4 T cells are required for the expression of LPR. Mice passively immunized with a saturating amount of IgE mAb to OVA displayed a strong early response, comparable to that of actively immunized animals, but they did not develop delayed ear swelling and eosinophil accumulation at the site of Ag injection.

These observations are in apparent contrast with a previous report [17] that mice passively sensitized with anti-hapten IgE Ab displayed LPR after skin painting with hapten. We propose that the discrepancy between this study and ours is in fact that hapten by itself may cause the release of various proinflammatory cytokines (TNF- α , IL-1, IFN- γ and IL-10) at the site of skin painting [18]. It is therefore possible that this hapten-induced inflammatory reaction may amplify and prolong the IgE Ab-dependent early response. We found that both IgE Ab and Ag-primed CD4 T cells are required for the induction of LPR in BALB/c nu/nu mice. This finding is consistent with that of Iwamoto *et al.* [19], who demonstrated the crucial role of CD4 T cells in the induction of an allergic inflammatory response in actively immunized animals. More recently, it was shown that mice lacking mature CD4 T cells failed to develop airway inflammation when challenged with sensitizing Ag [20].

Our data revealed that IgE Ab is necessary, although not sufficient, for the development of LPR. Although we have not analysed the precise mechanisms by which IgE Ab and Ag-primed CD4 T cells interact with each other, it is reasonable to propose that IgE-dependent mast cell activation favours the local recruitment of Ag-specific T cells, for example by up-regulating the expression of adhesion molecules on vascular endothelial cells. We also confirmed the important role of IL-4 in Th2 lymphocyte-mediated inflammation [13] by showing that pretreatment of actively immunized mice with anti-IL-4 mAb selectively blocked LPR without affecting the early response. Moreover, a similar suppression was obtained by local injection of soluble IL-4 receptor (not shown). It is of interest to note that the Ag-primed CD4 T cells employed in the transfer experiments retained the capacity to produce IFN- γ in that spleen cells of OVA-immunized animals released high levels of IFN- γ *in vitro* upon stimulation with OVA (data not detailed). However, *in vivo*, there was no increase but rather a decrease of IFN- γ expression at the site of Ag challenge.

The lack of IFN- γ production might be accounted for by the inhibitory effects of either mast cell-derived mediators

or Th2 cytokines (IL-4 and IL-10). Indeed, PGs are known to selectively inhibit the production of type 1 (IFN- γ , IL-2) but not type 2 cytokines [21], and the inhibitory effect of IL-4 and IL-10 on IFN- γ production has been well documented [22–24]. It would be of interest to examine the production and the role of IL-10 in the induction of LPR, given that IL-10 may be induced by inflammatory mediators like PGE2 and that it may be produced by keratinocytes [25]. As already mentioned, mast cells may store and release IL-4 [12] and most of the IL-4-containing cells identified by immunohistological staining of nasal mucosa of allergic individuals were proved to be mast cells [26].

In this study, we did not examine the cell source of IL-4 and our failure to detect increased IL-4 levels after Ag challenge of mice passively immunized with IgE Ab does not rule out the possibility that mast cell-derived IL-4 is actively involved in the induction of LPR. Further studies are clearly required to define the mast cell–T cell interactions necessary for the development of LPR. In summary, the present study strongly suggests that both IgE Ab and CD4 T cells are required for the induction of LPR which appears to be a typical type 2 inflammatory response in which IL-4 has an obligatory role.

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