

Original Article

In situ expression of interleukin-4, -5 and -6 in Peyer's patch from ovalbumin-sensitized BALB/c mice after oral challenge

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ABSTRACT

The aim of the present study was to analyze *in situ* expression of cytokines (i.e. interleukin (IL)-4, IL-5, IL-6 and interferon (IFN)- γ in the gut after oral challenge with ovalbumin (OVA) in mice intraperitoneally sensitized with OVA and aluminum). High anti-OVA IgE production was elicited after sensitization. We confirmed the anaphylactic response in the gut after oral challenge by evaluation of the edema of villi and marked eosinophil infiltration in the lamina propria. Immunohistochemical studies demonstrated the presence of IL-4-, IL-5- and IL-6-producing cells (PC) and a lack of IFN- γ -PC in Peyer's patch. The kinetics of IL-5-PC and eosinophil infiltration showed a good synchronization. The predominance of the type 2 helper T cell (Th2) cytokine may lead to the anaphylactic response and marked eosinophil infiltration in the gut in our system. It is possible that our system mimics the immunological events that occur in the gut in food allergy.

Key words: cytokines, eosinophils, food allergy, gut.

INTRODUCTION

Food is considered to be one of the important allergens in atopic diseases, such as atopic dermatitis (AD). Specific IgE antibodies against several food allergens

(e.g. egg white, milk and soy beans) are often elevated, particularly in children with AD. In food allergic colitis, a marked eosinophilia and an increase in IgE-containing mononuclear cells has been observed in the lamina propria.¹ Interleukin (IL)-4 and IL-5 mRNA expression in skin lesions of AD have been confirmed by several authors,^{2,3} suggesting that the cytokine profile is a critical factor in inflammatory events such as eosinophilia and the increase in serum IgE concentration. Thus far there are few reports concerning *in situ* cytokine expression in the gut. Xu-Amano *et al.* have elucidated the type 2 helper T cell (Th2) cytokine-releasing pattern in T cell clones obtained from Peyer's patch,⁴ but their investigations were designed to clarify the IgA-producing system.

The purpose of the present study was to analyze an *in situ* cytokine expression pattern in the gut after oral challenge with ovalbumin (OVA) in mice with high anti-OVA IgE production.

METHODS

Animals

Eight-week-old female BALB/c mice (CLEA Co., Tokyo, Japan) were used in this study.

Immunization

Mice were divided into two groups. Group 1 (G1) mice were immunized twice, at an interval of 2 weeks, with 1 μ g OVA in 3 mg aluminum, intraperitoneally, following the method of Tomoe.⁵ Group 2 (G2) mice underwent the same immunization protocol with 3 mg aluminum alone.

The serum IgE titers from sensitized mice were

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determined by passive cutaneous anaphylaxis (PCA), using 7-week-old male Sprague-Dawley rats (CLEA Co.). Sensitization was achieved by injecting 1:2 dilutions (beginning at 1:40) of mouse sera (0.05 mL) into the shaved back of the rats. After 48 h, rats received an i.v. injection of 1 mg OVA in 0.5 mL of 1% Evan's blue dye in phosphate-buffered saline (PBS). Rats were killed 10 min after i.v. injection and the endpoint titer was selected as the last dilution resulting in a diameter of blueing of ≥ 5 mm.

Challenge

In G1 and G2 mice, challenges were performed 2 weeks after the final sensitization by instilling 150 mg OVA in 0.5 mL PBS via an intragastric cannula. Animals were fasted 18–24 h before antigen provocation. As a control, oral challenges were performed in each group to PBS alone (control G1, control G2).

Antibodies

Rat anti-mouse IL-4 monoclonal antibody (mAb; IgG_{2b}, BVD4-1-D11), rat anti-mouse IL-5 mAb (IgG₁, TRFK5), rat anti-mouse IL-6 mAb (IgG₁, MP5-20F3) and rat anti-mouse IFN- γ mAb (IgG₁, XMG1.2) were obtained from Pharmingen (San Diego, CA, USA). For alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining, anti-rat IgG antibody and APAAP were purchased from DAKO (Glostrup, Denmark).

Samples and fixation of tissues

Jejuno-ileal samples and ileal Peyer's patch were taken from all groups at 1, 3, 6, 12 and 24 h and 3 days after challenge. For hematoxylin and eosin (HE) staining, samples were fixed in 10% formalin. For immunohistochemical studies, samples were snap frozen and stored at -80°C until use. To test eosinophil infiltration in this system, cryostat sections were stained with Wright-Giemsa.

Immunohistochemistry

After fixation in 4% paraformaldehyde in PBS, sections were washed in Tris-buffered saline (TBS), blocked with 2% normal goat serum in TBS for 1 h at room temperature to avoid non-specific staining and incubated overnight at 4°C with first antibody (rat anti-mouse IL-4, IL-5, IL-6 or IFN- γ mAb) diluted 1:50 in 5% bovine serum albumin (BSA) in TBS (BSA/TBS). The strong endogenous

alkaline phosphatase staining expressed by intestinal epithelial cells was successfully blocked with 20% acetic acid.⁶ Subsequently, sections were incubated with anti-rat IgG antibody (DAKO; 1:40 diluted in 5% BSA/TBS, 45 min, room temperature) and APAAP raised by rat (DAKO; 1:50 diluted in 5% BSA/TBS, 45 min, room temperature). Finally, reactions were developed using a New Fuchsin kit (DAKO).

As a positive control, lymphocytes obtained from normal mouse spleen were stimulated *in vitro* by ionomycin (Calbiochem, San Diego, CA, USA) in combination with phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St Louis, MO, USA) according to the method of Andersson *et al.*⁷ The cytocentrifuge smears obtained from the 48 h culture were stained with each anti-cytokine mAb used in this study.

As a negative control, rat myeloma IgG₁ and IgG_{2b} (Zymed, San Francisco, CA, USA) were reacted instead of the first antibodies.

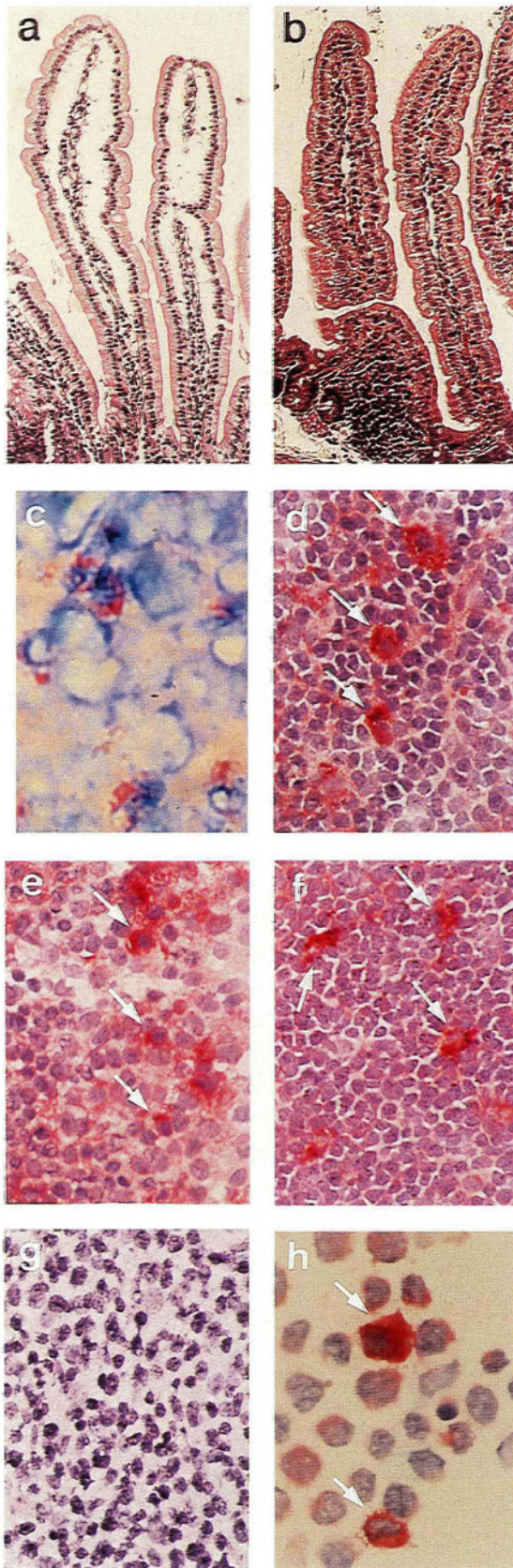
Quantification

Evaluation of positive-stained cells was performed according to the method of Bjerke *et al.*⁸ Briefly, an ocular grid was used to count stained cells in the gut. Subepithelial regions were divided into areas of 0.008 mm² and data are expressed as mean numbers of positive cells per area. For eosinophil counting in tissues, nine representative well-orientated crypts and villi per section were evaluated. The results were analyzed by a Student's *t*-test for comparison using StatView 4.0 software (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

Passive cutaneous anaphylaxis showed positive at a 640-fold dilution on average in G1 mice, while all sera from G2 mice were negative. We confirmed the anaphylactic response in the gut after oral challenge only in G1 mice by evaluation of the edema of the villi (Fig. 1a,b) and marked eosinophil infiltration (Fig. 1c) in the intestinal lamina propria.

In cytokine staining, IL-4-, IL-5- and IL-6-PC, but not IFN- γ -PC were found mainly in the dome region in Peyer's patch in G1 mice (Fig. 1d–f). In the intestinal lamina propria, a few scattered cytokine-producing cells were present. No staining with rat myeloma IgG₁ (Fig. 1g) or rat myeloma IgG_{2b} (data not shown) in either the Peyer's patch or in the intestinal mucosa was observed.

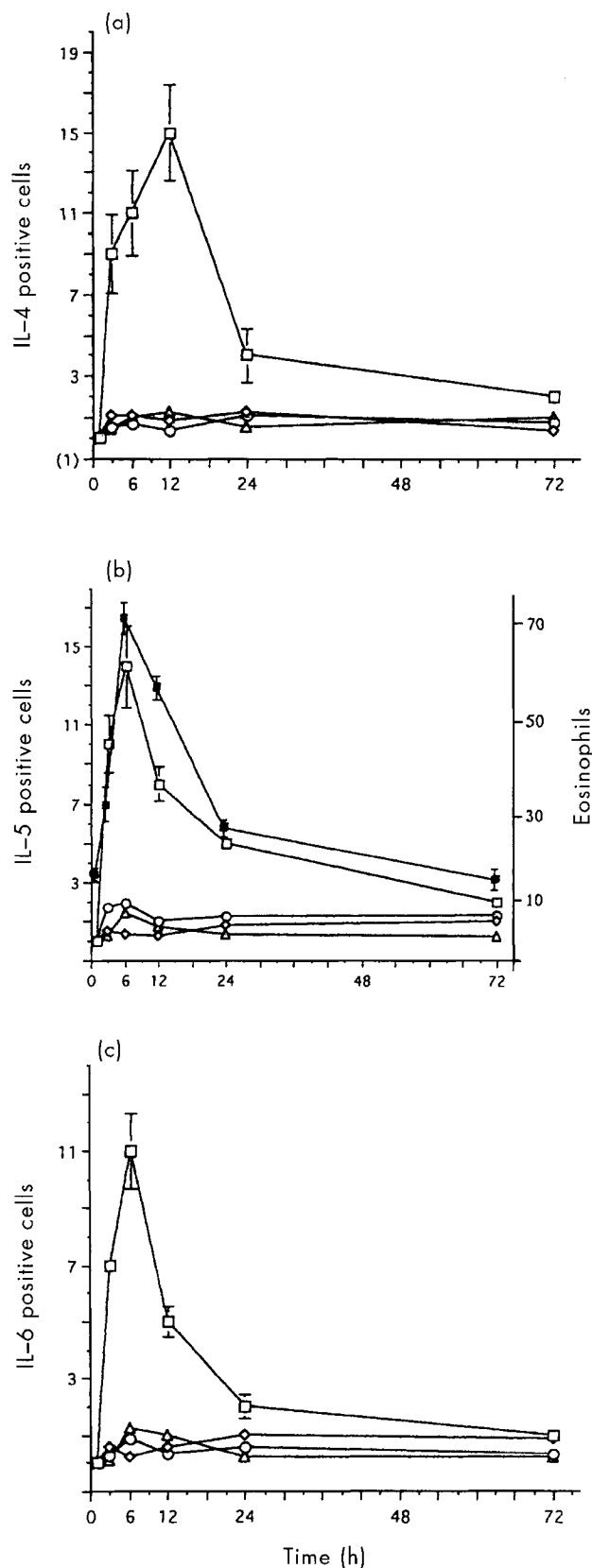


In G1 mice, IL-4-PC appeared from 3 h after oral challenge and peaked at 12 h (Fig. 2a). Interleukin-5-PC were found mainly in the same area as IL-4-PC and peaked at 6 h after challenge. The kinetics of the appearance of IL-5-PC and eosinophil infiltration showed a good correlation (Fig. 2b). Interleukin-6-PC also peaked at 6 h (Fig. 2c). In all the other groups (G2, control G1 and control G2 mice), no significant number of IL-4-, IL-5- or IL-6-PC were observed in the gut (Fig. 2a–c). No or very few IFN- γ -PC were observed in the Peyer's patch and lamina propria in any of the experimental groups (data not shown). The cytocentrifuge smears of ionomycin- and PMA-stimulated lymphocytes from normal mouse spleen were stained with the first antibodies used in the experiment (anti-mouse IL-4, IL-5, IL-6 and IFN- γ mAb), following the same staining method. The stimulated lymphocytes expressed all the cytokines. Some of the lymphocytes produced IFN- γ as a result of the stimulation (Fig. 1h). These findings support the lack of IFN- γ -PC in the gut in our system.

DISCUSSION

The predominance of Th2 cells and a marked eosinophil infiltration have been reported in lesions of atopic disorders, such as late-phase reaction,⁹ bronchial asthma,¹⁰ allergic rhinitis¹¹ and AD.² However, the cytokine expression pattern and even the immunological events in the gut in food allergy remain to be clarified following the ingestion of offending food. In the present study we demonstrated an edema of the villi and a marked eosinophil infiltration in the gut after oral challenge with OVA in sensitized mice producing a high IgE titer to OVA. Thus far, there have been several reports^{12–14} demonstrating oral challenge-induced

Fig. 1 (a) Edema of villi 3 h after oral challenge with ovalbumin (OVA) in group 1 mice. (b) No edema of villi 3 h after oral challenge with phosphate-buffered saline in control group 1 mice. (c) Marked eosinophil infiltration in the lamina propria 6 h after oral challenge with OVA in group 1 mice. Cytokine-producing cells in the mixed cell zone of Peyer's patch 6 h after oral challenge with OVA in group 1 mice (d–g). Interleukin (IL)-4- (d), IL-5- (e) and IL-6-producing cells (f) are indicated by arrows. (g) No staining was observed by isotype-matched control antibody (rat myeloma IgG₁). (h) Interferon- γ -producing cells (arrows) on a cytocentrifuge smear of ionomycin and phorbol 12-myristate 13s-acetate-stimulated spleen cells from normal mouse (original magnification $\times 250$).



intestinal anaphylaxis (e.g. mucosal edema), increased permeability of intestinal tissue and goblet cell mucus release in mice acquiring a high IgE titer against the antigen as a result of intraperitoneal sensitization. The intestinal anaphylaxis may be IgE dependent in view of the finding that reduction of small intestinal water and electrolyte absorption at oral challenge was related to the PCA titer¹² and that goblet cell mucus release was also observed in mice passively sensitized with serum containing a high IgE titer against the antigen after the oral challenge.¹⁴ The cytokine profile of our system showed a Th2 cell pattern; namely, IL-4-, IL-5- and IL-6-PC were observed in Peyer's patch in the absence of IFN- γ expression. In our system, OVA-sensitized mice showed good IgE production against OVA and an edema of the villi and marked eosinophil infiltration in the gut were induced by oral challenge. Furthermore, good synchronization of eosinophil infiltration and the appearance of IL-5-PC was observed. These inflammatory events can be explained by the actions of Th2 cytokines.

No staining was observed by isotype-matched immunoglobulins (rat myeloma IgG₁ and rat myeloma IgG_{2b}) and staining of cytokine-positive cells showed a cytoplasmic pattern. This suggests that positive cells produce the cytokine *in situ* following oral challenge.

The cytokine-producing cells in Peyer's patch were found mainly in the mixed-cell zone of the Peyer's patch, the so-called 'T cell area' beneath the dome epithelium. This zone is considered to be the area where antigens are initially trapped and then presented to T lymphocytes in Peyer's patch. Even though we did not identify cytokine-producing cells in the biopsies, their localization and nuclear shape indicate that most were T cells. Mast cells¹⁵ and eosinophils³ have been reported to produce a variety of cytokines, including Th2 cytokines (e.g. IL-4, IL-5 and IL-6). Tanaka *et al.*³ demonstrated both IL-5 mRNA and protein in eosinophils infiltrating the skin in AD. The

Fig. 2 Kinetics of cytokine-producing cells in Peyer's patch from orally challenged mice. After staining, the number of cytokine-producing cells was quantitated as described in Methods and is expressed as the mean number of positive cell per 0.008 mm² area. The data are the mean $\pm$ SD of three different mice from each group. (a) Interleukin (IL)-4-producing cells; (b) kinetics of eosinophil infiltration and IL-5-producing cells; (c) IL-6-producing cells. (□), group 1; (○), group 1 control; (◇), group 2; (△), group 2 control; (■), eosinophils (group 1).

possibility remains that some of the cytokine-producing cells were mast cells and/or eosinophils.

However, IgA production is also considered to be controlled by Th2. Interleukin-4, IL-5 and IL-6 are known to stimulate IgA secretion from B cells. Xu-Amano *et al.*¹⁶ have reported that oral priming with tetanus toxoid (TT) and cholera toxin results in Th2 cytokine expression and TT-specific IgA production in lymphocytes obtained from Peyer's patch and spleen by simulation with TT-coated microspheres. At present, the differences in the mechanism of production of IgE and IgA are poorly understood. Th2 cytokines may be a common regulating factor considering the finding that oral immunization with keyhole limpet haemocyanin (KLH) and cholera toxin led to both IgA and IgE production against KLH in lamina propria, especially at an early stage.¹⁷ It may be that some factor (e.g. transforming growth factor- β , which is known to be an IgA class-switching factor) may be crucial in the IgA producing system.

In conclusion, our work has demonstrated the presence and kinetics of IL-4-, IL-5- and IL-6-PC and a lack of IFN- γ -PC in the gut after oral challenge in OVA-sensitized mice. The predominance of Th2 cells in this system may lead to high IgE production, an edema of the villi and a marked eosinophil infiltration after oral challenge. These events in this system may be similar to those occurring in the gut of patients with food allergy.

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