

B cell mitogenic activity of house dust mite, *Dermatophagoides farinae*, antigens

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Abstract

The effect of mite antigens on murine and human lymphocytes was studied in vitro. Antigens prepared from *Dermatophagoides farinae* feces and bodies stimulated normal murine spleen cells to proliferate in a dose-dependent manner. The responder cells are B cells, because the response was reduced by the treatment of spleen cells with anti-immunoglobulin antibody and complement, but not with anti-Thy 1 antibody and complement. Furthermore, nylon column-purified T cells did not respond. The stimulation of B cells with mite antigens was not due to the contamination of lipopolysaccharide, a representative B cell mitogen, because C3H/HeJ spleen cells which are low responders to lipopolysaccharide could respond to mite antigens. These antigens induced not only proliferative response of murine B cells, but also immunoglobulin production. By gel-filtration column chromatography, the active fractions were eluted around the molecular weight of 150–155 kDa. Furthermore, mite antigens also stimulated human B cells to proliferate and to produce immunoglobulin. All these results suggest that mite antigens are a potent B cell mitogen and this activity might concern the induction of allergic reaction.

Keywords: *Dermatophagoides farinae*; B cell proliferation; Ig production; Purified mite antigen

1. Introduction

House dust mites, *Dermatophagoides* (*D.*) *farinae* and *D. pteronyssinus*, are major allergens for allergic disorders such as asthma, eczema and rhinitis [1]. The characterization of mite antigens has been made by biochemical and immunological methods and several antigens such as *Der f* 1, *Der f* 2 and *Der f* 6 from *D. farinae* and their equivalents from *D. pteronyssinus* have been purified to homogeneity [2–4]. *Der f* 1, *Der f* 3 and *Der f* 6 have protease activity [5–7]. Recently, several investigators have cloned cDNAs coding for mite antigens such as *Der f* 1, *f* 2, *p* 1 and *p* 2 from *D. farinae* and *D. pteronyssinus* [8–14]. Our group also has characterized mite antigens from *D. farinae* [15–17] and cloned cDNAs coding for new allergens [18–21]. However little information is available on the biological

activities of these mite antigens. In this communication, we studied the effect of mite antigens on murine and human lymphocytes in vitro.

2. Materials and methods

2.1. Mice

C3H/HeN and C3H/HeJ mice were obtained from Seiwa Experimental Animal Co. (Yoshitomi, Ohita, Japan) and maintained on purina rodent chow and water ad libitum. Both male and female mice were used at 6–8 weeks of age.

2.2. Mite antigens

D. farinae were cultured on purina rodent chow (Oriental Yeast Co., Tokyo, Japan) at 37°C for 30 days. A whole mite culture was suspended in a satu-

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rated NaCl solution and centrifuged at $1400 \times g$ for 30 min. The soluble fraction was used as feces antigen. The floating mites were collected by filtration and ground with quartz in 10 mM phosphate-buffered saline (PBS) and centrifuged. The solubilized fraction was used as body antigen. Both antigen fractions were dialyzed and lyophilized. The freeze-dried extracts were referred to as Dff and Dfb, respectively [16].

2.3. Purification of mite antigens

Purification of mite antigens was followed according to our previous report [15]. Dff was applied to a UF-20C ultrafiltration apparatus (Tosoh Co., Shin-Nanyo, Japan) equipped with 20 sheets (11×14 cm) of UF-20S-10PS (10^4 M_r -cut off) filters (Tosoh) to eliminate small molecular weight antigens. The fraction retained in the apparatus was dialyzed and lyophilized as a high-molecular-size fraction (HM) of Dff. HM (1 g) was applied to an Ultrogel AcA54 column (4.4×100 cm, IBF, France) and eluted with 0.9% NaCl at a flow rate of 120 ml/h. The fraction was monitored, with an absorbance at 280 nm, for the amount of saccharide and protein by using the phenol sulfuric acid method and Lowry method [22], respectively. HM was divided into four fractions; HM-1, -2, -3 and -4 (Fig. 3a). HM-1 (500 mg) was applied to a Sepharose CL-6B column (4.4×70 cm, Pharmacia Biotechnology, Uppsala, Sweden) and fractionated into three fractions; HM1-1, 1-2 and 1-3 (Fig. 3b). HM1-2 (200 mg) was further applied to a DEAE-Toyopearl 650S column (2.6×10 cm, Tosoh) equilibrated with 10 mM phosphate buffer, pH 6.0. The column was washed with three bed volumes of the same buffer and antigens were eluted stepwise with 10 mM phosphate buffer containing 0.1, 0.3 and 1 M NaCl (Fig. 3c).

2.4. Assay of proliferative response

A spleen cell suspension was prepared from normal mice and was treated with 0.83% NH_4Cl in 0.017 M Tris buffer, pH 7.2, to lyse red blood cells. Spleen cells (2×10^5) were cultured with several concentrations of mite antigens, concanavalin A (Con A, 10 $\mu\text{g}/\text{ml}$, EY Laboratories, San Mateo, CA, USA), lipopolysaccharide from *Escherichia coli* (LPS, 10 $\mu\text{g}/\text{ml}$, Sigma Chemical Co., St. Louis, MO, USA) or *Staphylococcus aureus* Cowan I (SAC, 0.01%, Funakoshi Yakuhin Co., Tokyo, Japan) in 0.2 ml of Eagle-Hanks amino acid medium described by Corradin et al. [23] containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY, USA) in wells of flat-bottomed microtiter culture plates (Falcon 3072, Becton Dickinson Co., Oxnard, CA, USA) at 37°C for 3 days in 5% CO_2 and 95% air. The cells were labeled during the last 18 h with 18.5 kBq of tritiated thymidine ($[^3\text{H}]\text{TdR}$,

specific activity, 222 GBq/mmol, Amersham International plc, Buckinghamshire, UK) and were harvested with the aid of a semi-automated cell harvester (Abe Kagaku Co., Chiba, Japan). The amount of radioactivity incorporated into DNA in lymphocytes was measured with a liquid scintillation spectrophotometer [24]. The results are expressed as the mean counts per minute (counts/min) of triplicate cultures with standard error.

2.5. Fractionation of spleen cells

Spleen cells were treated with $100 \times$ diluted anti-mouse Thy 1.2 monoclonal antibody (Olac 1976, Blackthorn Biester, Oxon, UK) or $20 \times$ diluted rabbit anti-mouse immunoglobulin (Ig) serum (Cedarlane Laboratories, Hornby, Canada) and $6 \times$ diluted rabbit complement (C, Cedarlane Laboratories) at 37°C for 1 h to obtain B cell- and T cell-enriched fractions, respectively. After the treatment, Thy 1-positive cells and Ig-positive cells decreased to less than 1 and 5%, respectively, as detected by flow cytometry using fluoresceinated antibody, while the untreated spleen cells contained about 40% Thy 1-positive cells and 40% Ig-positive cells. Furthermore, T cells were purified by passing the spleen cells through a nylon wool column as described previously [25]. After the nylon wool column-passing, Ig-positive cells decreased to less than 1%.

2.6. Assay of Ig production by spleen cells

The production of Ig by spleen cells was detected by sandwich enzyme-linked immunosorbent assay (ELISA). Spleen cells ($1 \times 10^6/\text{ml}$) were cultured with Dfb, Dff, LPS or SAC in 24-well culture dishes (Falcon 3002) for 7 days and then the culture supernatant was harvested by centrifugation. For the assay of total IgG, a flat-bottomed microtiter plate (Sumitomo Bakelite Co., Tokyo, Japan) was coated with sheep anti-mouse IgG (5 $\mu\text{g}/\text{ml}$, The Binding Site Co., Birmingham, UK) at 37°C for 2 h and at 4°C for overnight. After blocking with 1% bovine serum albumin in PBS, the culture supernatant was added to the plate and incubated at 37°C for 1 h. After washing with PBS, peroxidase-conjugated sheep anti-mouse IgG antibody diluted $800 \times$ (The Binding Site Co.) was added to the plate and incubated at 37°C for 1 h. After washing with PBS, *o*-phenylenediamine dihydrochloride (5.5×10^{-4} M, Wako Jyunyaku Co., Kyoto, Japan) in 0.01% H_2O_2 solution was added as a substrate and incubated at room temperature for 30 min. The reaction was stopped with 8 M H_2SO_4 and the absorbance at 490 nm was measured by Immuno Reader NJ-2000 (Japan Intermed Co., Tokyo, Japan). Purified murine IgG was used as a standard. The results are expressed as the mean ng/ml of IgG produced by 10^6 spleen cells in triplicate cultures with standard error.

2.7. Culture of human peripheral blood lymphocytes (PBL)

PBL were prepared from heparinized peripheral blood of normal volunteers by centrifugation on a lymphocyte separation medium (Organon Teknica, Durham, NC, USA). For the preparation of T cell- and B cell-enriched fractions, PBL were treated with anti-CD3 or anti-CD20 antibody (Seikagaku Kogyo Co., Tokyo, Japan) and rabbit C (Cedarlane Laboratories) at 37°C for 1 h. After this treatment, CD3-positive cells and CD20-positive cells decreased to less than 2%, respectively, as detected by flow cytometry using fluoresceinated antibody, while the percentage of CD3-positive cells and CD20-positive cells of the untreated PBL was about 70 and 20%, respectively. The assay systems of proliferative response and Ig production were the same as those of murine spleen cells described above.

2.8. Statistics

All experiments were repeated at least three times and one representative result was shown in table form and graphically. Statistical analysis between control values and stimulated ones was performed by Student's *t*-test. A confidence level of $P < 0.05$ was considered significant [26].

3. Results

3.1. Mitogenic activity of mite antigens on murine spleen cells

At first, we studied the effect of mite antigens on the proliferative response of murine spleen cells. Crude mite antigens (Dfb and Dff) stimulated the proliferative response of normal spleen cells at concentrations of 1–100 $\mu\text{g/ml}$ in a dose-dependent manner (data not shown). In the following experiments, we mainly used 100 $\mu\text{g/ml}$ of the antigens. Fig. 1 shows the time course of proliferative responses of spleen cells stimulated with mite antigens. The peak response was observed in 2 to 3 days and then the response gradually decreased. Thus, in the following experiments, we assayed on day 3.

3.2. Responder cells are B cells

In order to determine the responder cells to mite antigens, we fractionated spleen cells using anti-Thy 1 or anti-Ig antibody and C. Control cells were treated with C alone. As shown in Fig. 2a, control cells responded well to Con A, T cell mitogen, LPS, B cell mitogen and mite antigens. Anti-Thy 1 antibody and C

treatment completely abrogated the Con A response, but the responses of LPS and mite antigens were better than that of the control cells. On the other hand, anti-Ig antibody and C treatment depleted the responses of LPS and mite antigens, but the Con A response was enhanced. Since the B cell depletion from spleen cells with anti-Ig antibody and C treatment was not complete, we next depleted B cells by using a nylon wool column. As shown in Fig. 2b, nylon-purified T cells alone weakly responded to Con A, LPS and mite antigens. The addition of mitomycin C (50 $\mu\text{g/ml}$, Kyowahakko Kogyo Co., Yokohama, Japan)-treated spleen adherent cells as accessory cells could restore the Con A response, but not the LPS and mite antigens responses. These data suggest that the responder cells to mite antigens are B cells but not T cells.

3.3. Mitogenic activity is not due to LPS

A representative B cell mitogen is LPS. A possibility is that the mitogenic activity of mite antigens may be LPS contaminated in the antigen preparation. Then, we studied the stimulation with mite antigens using spleen cells from C3H/HeJ mice which are low responders to LPS. As shown in Fig. 2c, C3H/HeJ spleen cells significantly responded to Con A and mite antigens but not to LPS. Furthermore, polymyxin B, an inhibitor of LPS response, did not reduce the response of mite antigens (data not shown). These results suggest that the mitogenic activity of mite antigens is not due to LPS.

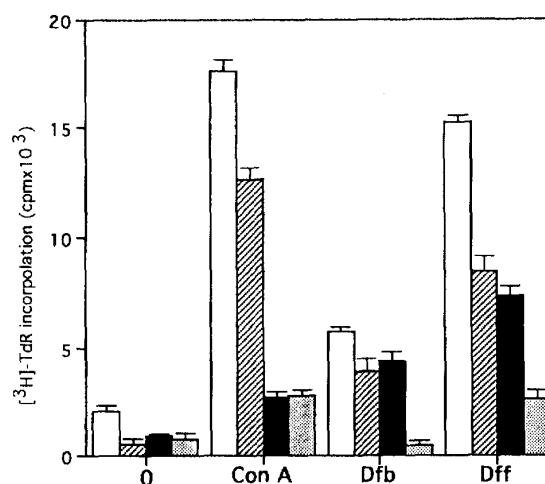


Fig. 1. Time course of proliferative response of spleen cells: 2×10^5 spleen cells from C3H/HeN mice were cultured in vitro with 100 $\mu\text{g/ml}$ Dfb, Dff or 10 $\mu\text{g/ml}$ Con A and harvested on days 2 (□), 4 (▨), 6 (■) and 8 (■). The cells were pulsed with 18.5 kBq of [³H]TdR for the last 18 h. [³H]TdR incorporated by spleen cells was counted by a liquid scintillation spectrophotometer. The results are expressed as the mean $\pm$ standard error counts/min in triplicate cultures.

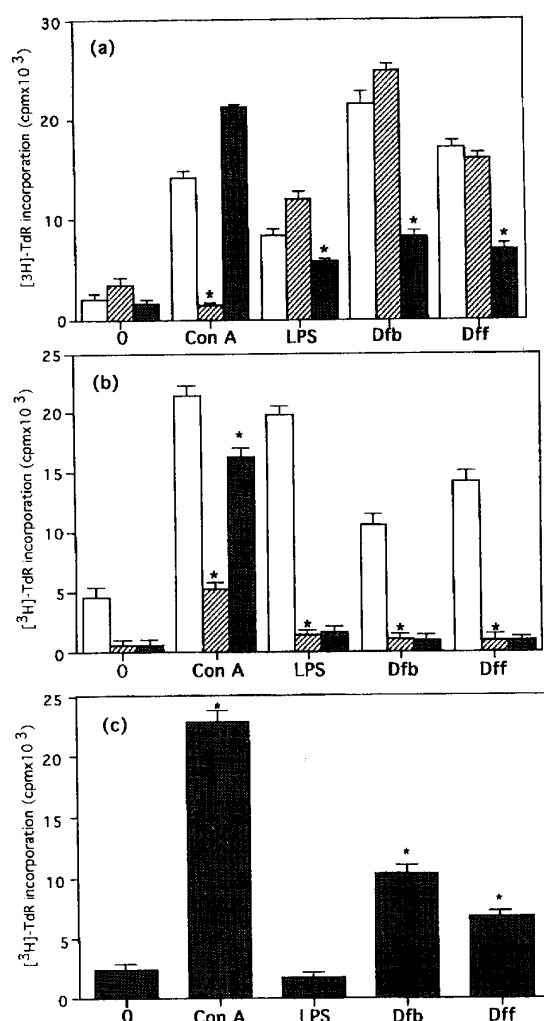


Fig. 2. (a) Responder cells are B cells for the stimulation with Dfb and Dff: 2×10^5 spleen cells treated with C alone ($\square$), anti-Thy 1 antibody and C ($\square$) or anti-mouse Ig and C ($\blacksquare$) were cultured with 10 $\mu\text{g}/\text{ml}$ Con A, LPS, 100 $\mu\text{g}/\text{ml}$ Dfb or Dff for 3 days and $[^3\text{H}]\text{TdR}$ incorporation was determined. *Significantly suppressed from the group treated with C alone ($P < 0.05$). (b) Nylon column-purified T cells did not respond to Dfb and Dff: 2×10^5 spleen cells ($\square$) or nylon column-purified T cells were cultured in the absence ($\square$) or presence ($\blacksquare$) of 10^5 mitomycin C-treated spleen cells as accessory cells with 10 $\mu\text{g}/\text{ml}$ Con A, LPS, 100 $\mu\text{g}/\text{ml}$ Dfb or Dff for 3 days and $[^3\text{H}]\text{TdR}$ incorporation was determined. *Significantly suppressed from the corresponding control group by nylon column-passing ($P < 0.05$). **Significantly enhanced by the addition of mitomycin C-treated spleen cells ($P < 0.05$). (c) C3H/HeJ spleen cells can respond to Dfb and Dff: 2×10^5 spleen cells from C3H/HeJ mice were cultured with 10 $\mu\text{g}/\text{ml}$ Con A, LPS, 100 $\mu\text{g}/\text{ml}$ Dfb or Dff for 3 days and $[^3\text{H}]\text{TdR}$ incorporation was determined. *Significantly stimulated ($P < 0.05$).

3.4. Purification of mite antigens

We purified mite antigens using gel-filtration and ion exchange column chromatography as shown in

Fig. 3. B cell mitogenic activity was fractionated into HM1-2 (M_r 150–155 kDa) by a Sepharose CL-6B column and eluted with 0.3 M NaCl by a DEAE column. Purified *Der f* 2 also had a mitogenic activity.

3.5. Mite antigens stimulate Ig production

Next, we studied the effect of mite antigens on the production of Ig. As shown in Table 1, mite antigens enhanced total IgG productions as well as LPS. These results suggest that mite antigens stimulate not only a proliferative response of B cells, but also polyclonal Ig production.

3.6. Mite antigens also stimulate human B cells

Finally we studied the effect of mite antigens on human lymphocytes. As shown in Fig. 4, mite antigens stimulated human lymphocytes to proliferate. This response was depleted after anti-CD20 antibody and C treatment of PBL, but not with anti-CD3 antibody and C treatment, suggesting that the responder cells to mite antigens in the human are B cells as is the case with murine lymphocytes. Mite antigens also stimulated the production of total IgG (Table 2). These results suggest that mite antigens activate human B cells as well as murine B cells.

4. Discussion

In this paper, we studied the effect of mite antigens on murine and human lymphocytes in vitro. *D. farinae* feces (Dff) and bodies (Dfb) antigens markedly stimulated normal murine lymphocytes to proliferate dose-dependently. The maximal proliferative response of mite crude antigens, Dfb and Dff, was observed at 100 $\mu\text{g}/\text{ml}$ (data not shown). This antigen concentration is a little bit higher than the concentration of other mitogens. The reason may be that Dfb and Dff are crude antigens, because the maximal response of purified mite antigens was observed at 10 $\mu\text{g}/\text{ml}$ on day 2 (data not shown). Nevertheless, the mitogenic activities of Dfb and Dff are very potent and the responses were sometimes comparable to those of Con A and LPS, representative mitogens.

The active fraction resided around the M_r of 150–155 kDa by gel-filtration column chromatography, and these fractions are known as glycoproteins. Several mite antigens are reported to be purified to homogeneity [2–4]. In fact, HM3 fractions contain *Der f* 2 and HM2 fractions contain *Der f* 6 by Western blot analysis (data not shown), but the proliferative response of murine lymphocytes stimulated with HM2 and HM3 was lower

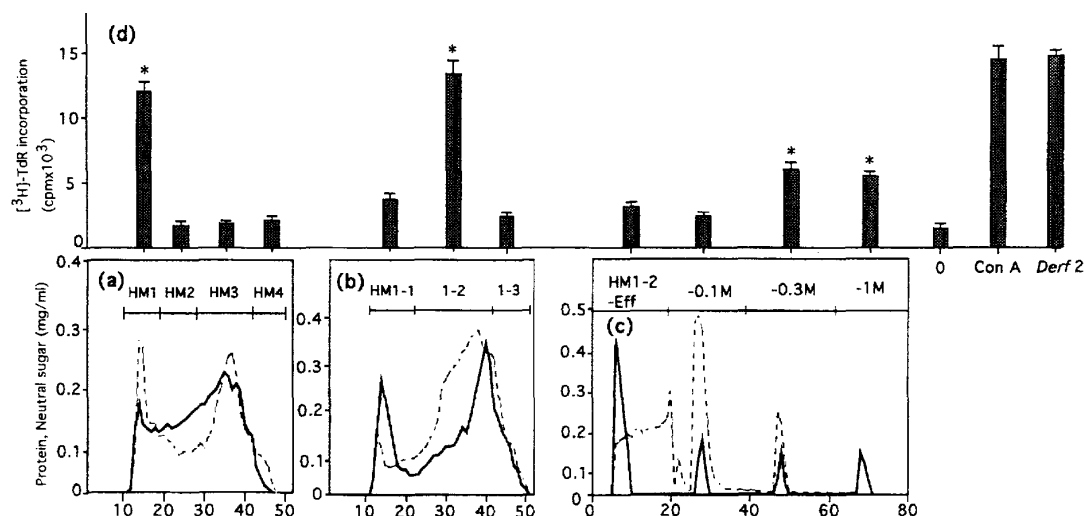


Fig. 3. High molecular size fractions of Df were fractionated on an Ultrogel AcA 54 column (a), Sepharose CL-6B column (b) and DEAE Toyopearl 650S column (c). The lines show protein (—) and neutral sugar (---) concentration. Abscissa shows fraction numbers. (d) Proliferative response of fractionated Df antigens on murine spleen cells: 2×10^5 spleen cells were cultured with $10 \mu\text{g/ml}$ of fractions or *Derf 2* for 3 days and $[^3\text{H}]\text{TdR}$ incorporation was determined. *Significantly stimulated ($P < 0.05$).

than that of HM1. Purified *Derf 2* also had a mitogenic activity (Fig. 3). However, HM1-2 fractions do not contain any corresponding mite antigens so far reported. The physicochemical nature of B cell mitogenic activity of HM1-2 fractions is under investigation.

Mite antigens also stimulated murine lymphocytes to produce Ig, especially IgG. Total IgE production was weak, and significant stimulation with mite antigens was not observed. This IgG production is independent on T cells, because Ig production was not affected by the treatment of lymphocytes with anti-Thy 1 antibody and C (data not shown). This is consistent with the phenomenon that mite antigens stimulate B cells to proliferative response. Mite antigens also stimulated human B cells to proliferate and to produce Ig. However, the responsiveness of murine lymphocytes is always much higher than that of humans. This may be explained by species difference or the source of

lymphocytes. In patients with mite allergy, specific IgG and IgE and non-specific IgE antibodies were elevated in their sera [27]. How can the relation of B cell mitogenic activity of mite antigens and the elevation of specific antibody be explained?

We observed that the proliferative response induced by mite antigens in vitro was much higher in mite allergen-sensitive patients and mite antigen-immunized

Table 1
Mite antigens stimulate immunoglobulin production by spleen cells

Spleen cells cultured with	IgG production ^a (ng/10 ⁶ cells)
Control	920 $\pm$ 86
LPS	6965 $\pm$ 113 ^b
Dfb	2108 $\pm$ 57 ^b
Dff	1406 $\pm$ 26 ^b

^a $10^6/\text{ml}$ spleen cells were cultured with $10 \mu\text{g/ml}$ LPS, $100 \mu\text{g/ml}$ Dfb or Dff for 7 days and IgG production in the culture supernatant was assayed by ELISA.

^bSignificantly enhanced ($P < 0.05$).

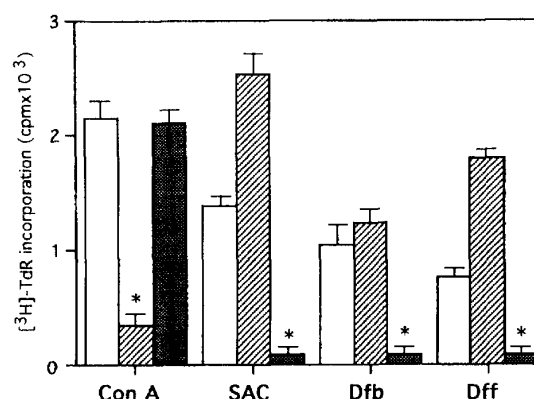


Fig. 4. *Dermatophagoides* antigens stimulate human B cells: 2×10^5 human peripheral blood lymphocytes treated with C alone ($\square$), anti-CD3 antibody and C ($\square$) or anti-CD20 antibody and C ($\blacksquare$) were cultured with $10 \mu\text{g/ml}$ Con A, 0.05% SAC, $100 \mu\text{g/ml}$ Dfb or Dff for 3 days and $[^3\text{H}]\text{TdR}$ incorporation was determined. The results were expressed as Δ cpm in which cpm of control group was subtracted from cpm of stimulated group. *cpm without mitogen is significantly suppressed from the group treated with C alone ($P < 0.05$).

Table 2

Mite antigens stimulate human lymphocytes to produce immunoglobulin

PBL	IgG production ^a (ng/10 ⁶ cells)
Control	219 ± 12
SAC	279 ± 20 ^b
Dfb	276 ± 18 ^b
Dff	270 ± 16 ^b

^a10⁶/ml lymphocytes were cultured with 0.05% SAC, 100 µg/ml Dfb or Dff for 7 days and IgG production in the culture supernatant was assayed by ELISA.

^bSignificantly enhanced ($p < 0.05$).

mice than in each normal control and this enhanced response is mediated by T cells (manuscript in preparation). These results suggest that mite antigens have two activities; non-specific B cell mitogenic activity and specific T cell priming activity. Previously we reported that sea squirt antigen, Hoya asthma-inducing antigen, has murine and human B cell-stimulating activity in vitro [28]. Thus, it can be considered that allergy-inducing antigens are intrinsically B cell mitogens. The role of non-specific B cell mitogenic activity of allergy-inducing antigens for specific B and T cell activation is under investigation.

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