

Human T cell responses to recombinant mite antigens of *Dermatophagoides farinae*

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SUMMARY

We studied T cell responses to four glutathione S transferase (GST)-fused mite antigens prepared in our laboratory using peripheral blood lymphocytes from mite-sensitive patients with bronchial asthma. Of the four recombinant antigens, purified GST-Mag3 had the strongest ability to cause patients' lymphocytes to proliferate, and its potency was almost comparable to that of crude mite bodies (Dfb) and faeces (Dff) antigens and a purified major antigen, *Der f* 2. The responder lymphocytes were mainly T cells, because the proliferative response was depleted by the treatment of lymphocytes with anti-CD3 antibody and complement, but not with anti-CD20 antibody and complement. The responsiveness of lymphocytes to GST-Mag3 correlated with that to *Der f* 2, but GST-Mag3 displayed slightly higher activity to stimulate lymphocytes than *Der f* 2. Simultaneously, the levels of Dff- and GST-Mag3-specific IgE antibodies correlated with the responsiveness of lymphocytes to GST-Mag3. These results suggest that Mag3 is a new valuable antigen for the response of T cell proliferation in mite-sensitive patients.

Keywords *Dermatophagoides farinae* recombinant antigen GST-fused protein T cell response

INTRODUCTION

Allergens from house dust mites, *Dermatophagoides farinae* and *D. pteronyssinus*, are major triggers for allergic disorders such as asthma, eczema, and rhinitis [1,2]. The characterization of mite antigens has been made by biochemical, immunological, and genetic engineering methods [1–3]. Recently, cDNAs coding for mite antigens, *Der* 1 [4,5], *Der* 2 [6,7], *Der* 3 [8,9], *Der p* 5 [10,11], *Der* 7 [12,13], and *Der p* 8 [14] have been cloned and recombinant antigens have become available. Among them, the proliferative responses of T cells to the major allergens, *Der* 1 and *Der* 2, were studied to identify T cell epitopes on these allergens using peripheral blood lymphocytes (PBL) from mite-sensitive patients [15,16].

We have also cloned four cDNAs coding for new allergens from a *D. farinae* library which were referred to as mite antigen gene (*mag*) 1, 3, 29, and 44 [17–21]. The recombinant products as well as the natural correspondences have been immunochemically characterized. The recombinant antigens could bind to IgE antibody in allergic sera with comparatively high frequencies. Especially, IgE-binding frequencies of Mag3 and Mag44 were comparable to those of the major allergens [19,21]. Mag29 is

nearly identical in sequence to heat shock protein (hsp) [20], while Mag44 has been identified as tropomyosin [21]. However, little information is available on the biological functions of the recombinant antigens.

In this paper, we studied T cell responses to these recombinant mite antigens prepared in our laboratory using peripheral lymphocytes from mite-sensitive patients with bronchial asthma.

PATIENTS AND METHODS

Subjects

Blood samples were obtained from 18 asthma patients with positive skin reaction or radioallergosorbent test (RAST) scores > 1, who came to the Third Department of Internal Medicine, Hiroshima Prefectural Hospital (Hiroshima, Japan) as regular outpatients, and 10 healthy volunteers insensitive to house dust mites.

Dermatophagoides antigens

Dermatophagoides farinae was cultured on purina rodent chow as previously described [22]. A whole mite culture was suspended in a saturated sodium chloride solution to separate mites. The soluble fraction was pooled as faeces antigen. The mites were ground with quartz in 10 mM PBS pH 7.2. The solubilized fraction was collected by centrifugation at 5000 rev/min as bodies antigen. Both antigen fractions were dialysed against PBS and lyophilized.

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The major allergen, *Der f 2*, was prepared in our laboratory according to the method of Yasueda *et al.* [23].

Preparation of recombinant fusion antigens

Recombinant antigens were purified as described previously [19–21]. Briefly, an expression plasmid carrying *mag1*, *mag3*, *mag29*, or *mag44* was constructed on a plasmid pGEX-2T (Pharmacia Biotech, Uppsala, Sweden) and transformed into *Escherichia coli* JM105. Isopropyl- β -D-thiogalactoside (IPTG)-induced cells in 2 l of culture were harvested and disrupted by sonication in PBS. Purified GST-Mag1 (65 kD), Mag3 (66 kD), Mag29 (43 kD) and Mag44 (63 kD) were obtained by passing through a glutathione Sepharose 4B (Pharmacia Biotech) column or by preparative disk gel electrophoresis (model NA-1800; Nippon Eido, Tokyo, Japan).

Preparation of human PBL

Heparinized human peripheral blood was centrifuged at 250 g for 30 min on a lymphocyte separation medium (Organon Teknica, Durham, NC). For the preparation of T cell- or B cell-enriched fractions, PBL were treated at 37°C for 1 h with anti-CD3 or anti-CD20 antibody (Seikagaku Kogyo, Tokyo, Japan) and rabbit complement (Cedarlane Labs, Ontario, Canada), respectively. After this treatment, CD3⁺ cells and CD20⁺ cells decreased to less than 2% as detected by flow cytometry using fluoresceinated antibody, while the percentage of CD3⁺ and CD20⁺ cells of the intact PBL were about 70% and 20%, respectively.

In some experiments, T cells were purified by passing through a nylon wool column, as described previously [24].

Assay of proliferative response of PBL

PBL (2×10^5) were cultured at 37°C for 6 days with recombinant fusion antigens (10 μ g/ml), *Dermatophagoides* antigens (10 μ g/ml), concanavalin A (Con A; 10 μ g/ml; EY Labs, San Mateo, CA), or *Staphylococcus aureus* Cowan I (SAC; 0.01%; Funakoshi Yakuhin, Tokyo, Japan) in wells of flat-bottomed microtitre culture plates (Falcon no. 3072; Becton Dickinson, Oxnard, CA) containing 0.2 ml of RPMI 1640 (Nissui Seiyaku, Tokyo, Japan) medium supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY) in 5% CO₂ and 95% air. The cells were labelled during the last 18 h with 18.5 kBq of tritiated thymidine (³H-TdR; specific activity 222 GBq/mmol; Amersham International plc, Aylesbury, UK) and were harvested with the aid of a semi-automated cell harvester (Abe Kagaku, Chiba, Japan). The amount of radioactivity incorporated into DNA in lymphocytes was measured with a liquid scintillation counter [25]. Results were expressed as the geometric mean of triplicate cultures with s.e.m. or stimulation index (SI), which was calculated as follows: SI = geometric mean ct/min in cultures stimulated with antigen / geometric mean ct/min in unstimulated culture.

Serum levels of total and antigen-specific IgE and antigen-specific IgG

The total IgE level in allergic sera was detected by sandwich ELISA [26]. Flat-bottomed microtitre plate (Sumitomo Bakelite, Tokyo, Japan) was coated at 37°C for 2 h or overnight at 4°C with sheep anti-mouse IgE antibody (5 μ g/ml; The Binding Site, Birmingham, UK), followed by blocking with 1% bovine serum albumin (BSA; Sigma, St Louis, MO) in PBS. The sera ($\times 10$) were added to the plate, incubated at 37°C for 1 h, and washed with PBS. The same procedures were repeated using peroxidase-conjugated

sheep anti-mouse IgE antibody ($\times 800$, The Binding Site). Citrate buffer (10 mM, pH 4.2) containing *o*-phenylenediamine dihydrochloride (5.5×10^{-4} M; Wako Junyaku, Osaka, Japan) and 0.01% H₂O₂ was added and incubated at room temperature for 30 min. The reaction was stopped with 4 M H₂SO₄ and absorbance at 490 nm was measured by Immuno Reader NJ-2000 (Japan Intermed, Tokyo, Japan).

Levels of antigen-specific IgG and IgE antibodies were also measured by ELISA. Dfb or Dff (10 μ g/ml) were coated at 37°C for 2 h or at 4°C overnight on flat-bottomed wells. After blocking, the allergic sera (IgG, $\times 300$; IgE, $\times 10$) in the wells were incubated at 37°C for 1 h. Wells were washed with PBS, followed by incubation of peroxidase-conjugated rabbit anti-human IgG ($\times 1000$; Organon Teknica) or biotin-labelled anti-human IgE ($\times 300$; KPL, Gaithersburg, MD) and peroxidase-conjugated streptavidin ($\times 300$; Bio Source, Camarillo, CA) at 37°C for 1 h. After reaction in 10 mM citrate buffer pH 4.2, containing 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS; Boehringer, Mannheim, Germany) and 0.03% H₂O₂, wells were measured at 405 nm with Immuno Reader NJ-2000.

The results are expressed as the mean values for duplicate assays.

Statistical analysis

All experiments were repeated at least three times and one representative result is shown as a table and figure. Statistical analysis was performed by Student's *t*-test. A confidence level of $P < 0.05$ was considered significant [27].

RESULTS

Activities of new recombinant mite antigens to stimulate lymphocytes from mite-sensitive patients

The proliferative response of PBL from a representative mite-sensitive patient, when new recombinant mite antigens were used as stimulants, is depicted in Fig. 1. Among four recombinant mite antigens, GST-Mag1 and GST-Mag3 significantly stimulated the lymphocytes to proliferate. Simultaneously, stimulation of lymphocytes with *Der f 2* or Dfb resulted in their proliferation, while

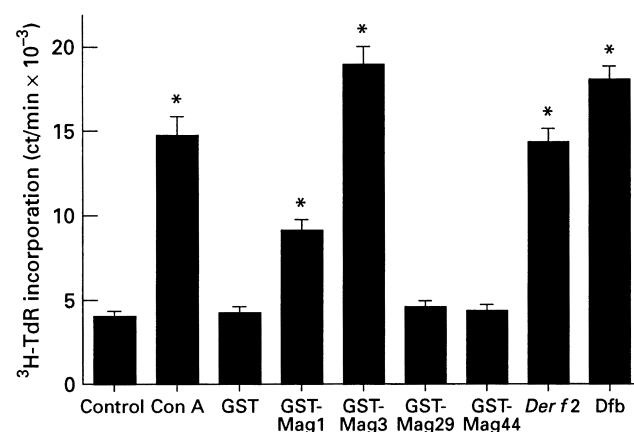


Fig. 1. Stimulating activity of mite antigens on human peripheral blood lymphocytes (PBL). PBL (2×10^5) from a mite-sensitive patient were cultured *in vitro* with 10 μ g/ml concanavalin A (Con A) or mite antigens for 6 days. The lymphocytes were pulsed with 18.5 kBq of ³H-TdR for the last 18 h. ³H-TdR incorporated into lymphocytes was counted by a liquid scintillation counter. Results are expressed as mean $\pm$ s.e.m. of triplicate cultures. *Significantly stimulated from control ($P < 0.05$).

GST-Mag29 and GST-Mag44 hardly stimulated proliferation of the lymphocytes as well as GST. From the result, we used mainly GST-Mag3 as a potent stimulant in the following experiments. The peak response in proliferation of lymphocytes from a patient stimulated with mite antigens was observed on day 6 and then gradually decreased (data not shown). Thus, in the following experiments, we assayed on day 6.

Responder lymphocytes to mite antigens are T cells

To determine which cells respond to mite antigens, T cells or B cells were enriched by the treatment of PBL from an allergic patient with anti-CD20 or anti-CD3 antibody and complement, respectively. Control cells were treated with complement alone. As shown in Fig. 2, control cells responded well to Con A (T cell mitogen), SAC (B cell mitogen), Dfb, and GST-Mag3. Treatment of lymphocytes with anti-CD3 antibody and complement abrogated Con A and mite antigen responses, but not the SAC response. On the other hand, treatment of lymphocytes with anti-CD20 antibody and complement depleted the SAC response, but not the Con A and mite antigen responses.

Furthermore, nylon-purified T cells from a mite-sensitive patient responded to mite antigens only in the presence of accessory cells (data not shown). These results suggest that the responder lymphocytes to mite antigens are T cells but not B cells.

Comparison of proliferative responses of lymphocytes from mite-sensitive patients with those from healthy subjects

GST-Mag3 stimulated specifically lymphocytes from mite-allergic patients or normal subjects to proliferate (Fig. 3). Additionally, proliferation responses of mite-sensitive and mite-insensitive lymphocytes ($n = 5$ and $n = 4$, respectively) were examined as reference. When mite-sensitive lymphocytes ($n = 18$) were compared with normal lymphocytes ($n = 10$) as to responsiveness to mite antigens, the mean SI values of the former were significantly higher than those of the latter ($P < 0.01$). The responsiveness of

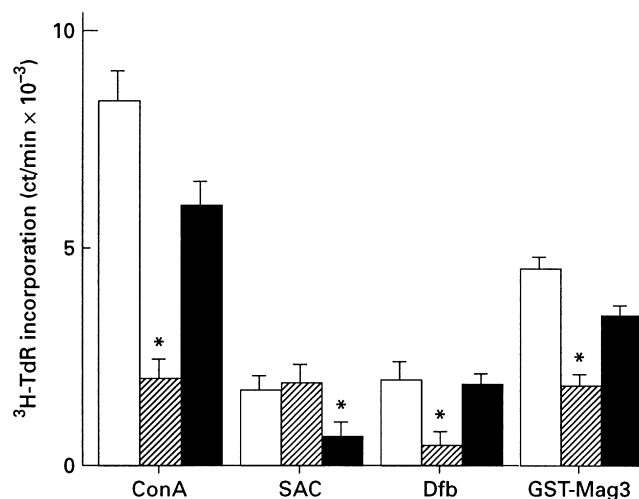


Fig. 2. Responder lymphocytes are T cells for stimulation with mite antigens. Peripheral blood lymphocytes (PBL; 2×10^5) treated with C alone (□), anti-CD3 antibody and C (▨), or anti-CD20 antibody and C (■) were cultured with $10 \mu\text{g/ml}$ concanavalin A (Con A), 0.01% *Staphylococcus aureus* Cowan I (SAC), $10 \mu\text{g/ml}$ Dfb, or $10 \mu\text{g/ml}$ GST-Mag3 for 6 days and ^3H -TdR incorporation was determined. *Significantly suppressed from the group treated with C alone ($P < 0.05$).

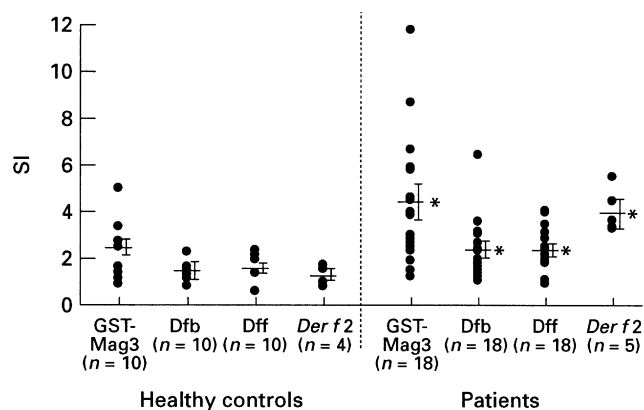


Fig. 3. Responsiveness of lymphocytes from mite-sensitive asthmatic patients and mite-insensitive subjects to mite antigens. Peripheral blood lymphocytes (PBL; 2×10^5) from mite-sensitive patients and normal subjects were cultured *in vitro* with $10 \mu\text{g/ml}$ mite antigens for 6 days and ^3H -TdR incorporation was determined. Results are expressed as stimulation index (SI). *Significantly enhanced by mite-sensitive patients compared with healthy controls ($P < 0.05$).

mite-sensitive lymphocytes stimulated with GST-Mag3 correlated to that with Der f2 ($r = 0.89$) (Fig. 4). Moreover, the SI values to GST-Mag3 were always higher than those to Der f2 (slope = $0.62 < 1$, $P < 0.05$). These results demonstrate that Mag3 displays potent ability to stimulate the proliferation of mite-specific T lymphocytes.

Correlation of specific IgG and IgE levels in mite-sensitive sera to the responsiveness of lymphocytes from mite-sensitive patients

Finally, we studied a correlation of the responsiveness of lymphocytes to GST-Mag3 with the levels of the antigen-specific IgE and IgG antibodies. The levels of Dff- and GST-Mag3-specific IgE antibodies correlated to the SI values to GST-Mag3 ($r = 0.55$ and 0.50 , respectively, $P < 0.05$) (Fig. 5b,c). However, a correlation

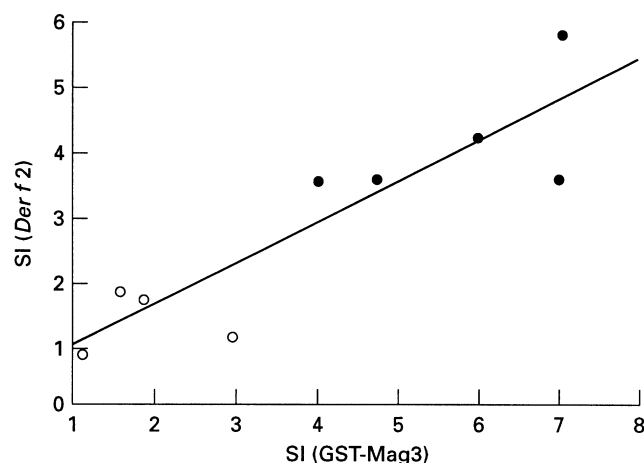


Fig. 4. Correlation between proliferative response of PBL to Mag3 and Der f2. Peripheral blood lymphocytes (PBL; 2×10^5) from mite-sensitive patients ($n = 5$, ●) and non-sensitive controls ($n = 4$, ○) were cultured *in vitro* with $10 \mu\text{g/ml}$ mite antigens for 6 days and ^3H -TdR incorporation was determined. The coefficients of regression and correlation for stimulation index (SI) values of GST-Mag3 versus those of Der f2 were determined to be 0.62 and 0.89 , respectively.

was not observed between the levels of Dfb-specific or total IgE antibodies and the SI values to GST-Mag3 ($r = 0.33$ or $r = 0.35$) (Fig. 5a,d). Any correlation between the SI values to Mag3 and the levels of Dfb- or Dff-specific IgG antibodies was not estimated ($r = 0.06$ or $r = 0.01$, respectively) (data not shown). These results indicate that Mag3 induces significant T cell responses and may be responsible for specific IgE responses as well.

DISCUSSION

In this paper, we studied the stimulating activity of four recombinant mite antigens on PBL from allergic patients sensitive to mites. Recombinant mite antigens, Mag1, Mag3, and Mag44, could bind to IgE in allergic sera with considerable high frequencies, while Mag29 could react to the IgE to a lesser extent. Furthermore, these antigens have no sequential homology with previously reported mite antigens such as *Der 1*, *Der 2*, *Der 3*, *Der p 5*, *Der 7*, and *Der p 8* [4–14]. Although GST-Mag29, GST-Mag44, and GST exerted no ability to promote proliferative responses of lymphocytes, GST-Mag1 and GST-Mag3 showed a distinguishable characteristic that remarkably stimulated lymphocytes from patients to proliferate in the same manner as crude mite antigens, Dfb and Dff, and a purified mite antigen, *Der f2*. It is noteworthy that GST-Mag44 could not activate T lymphocytes from mite-sensitive patients, although Mag44 (or GST-Mag44) has been found to have as high IgE-binding activity on immunoblots as the major allergens, *Der f1* and *Der f2*. The discrepancy remains to be solved.

GST-Mag1 and GST-Mag3 also had stimulating activity on lymphocytes from normal subjects. These data confirm our previous

report [22] that crude mite antigens had mitogenic or priming activity on normal murine and human B or T lymphocytes, respectively. Furthermore, the responsiveness of lymphocytes from allergic patients to mite antigens was always higher than that of normal lymphocytes. This is consistent with the reports of O'Hehir *et al.* [28] and Upham *et al.* [29], who demonstrated the antigen-specific responses in mite-sensitive patients. The enhanced activation of the lymphocytes should reflect upon the proliferative response for the antigen-specific T cells, because this enhanced response was depleted by treatment of the lymphocytes with anti-CD3 antibody and complement, and nylon-purified T cells responded to mite antigens in the presence of accessory cells.

The responsiveness of T cells to GST-Mag3 correlated with that to a major allergen, *Der f2*, but the SI values to Mag3 were always higher than those to *Der f2*. O'Brien *et al.* reported that the proliferative response of T cells to affinity-purified *Der p 1* paralleled that to affinity-purified *Der p 2* [30]. Our study using recombinant antigens presented here appended their data and suggests that reactivity to several mite antigens could be coordinately regulated. O'Brien *et al.* [30], O'Hehir *et al.* [31], Hiratani *et al.* [32], and Rawle *et al.* [33] reported that T cell responses to *Der p 1* and *Der p 2* correlated with total IgE levels in house dust mite-sensitive patients. We also studied the relationship between T cell responses and antibody levels in allergic patients and found that there is a correlation between the levels of Dff-specific IgE or GST-Mag3-specific IgE antibody in patients and responsiveness to Mag3. The results presented here suggest that the new mite antigen, especially Mag3, is useful for the detection of mite-sensitive patients and for the study of T cell activation mechanism

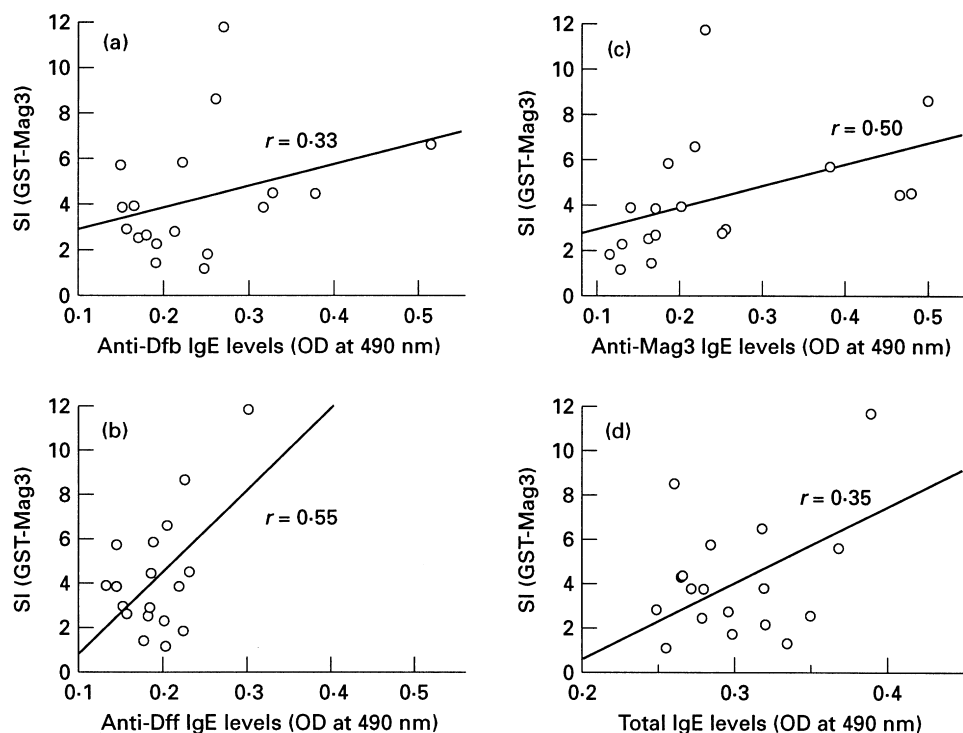


Fig. 5. Correlation between proliferative responses of peripheral blood lymphocytes (PBL) to GST-Mag3 and levels of anti-Dfb IgE antibody (a), anti-Dff IgE antibody (b), anti-GST-Mag3 IgE antibody (c), or total IgE antibody (d). PBL (2×10^5) from mite-sensitive patients were cultured *in vitro* with $10 \mu\text{g/ml}$ GST-Mag3 for 6 days and ^3H -TdR incorporation was determined. Results are expressed as stimulation index (SI) values. Levels of anti-Dfb IgE, anti-Dff IgE, anti-GST-Mag3 IgE, and total IgE antibodies in sera from patients were determined by ELISA. Results are expressed as absorbance at 490 nm. Coefficients of correlation for a, b, c, and d were estimated to be 0.33, 0.55, 0.50, and 0.35, respectively.

by mite antigens. In order to determine which determinants T cells recognize, deletion mutant of *mag1* and *mag3* are being prepared by genetic engineering techniques.

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