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Effect of Mite Antigens on Antigen Presenting Cells/ Macrophages in Mice

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Abstract: The effect of mite antigens on murine lymphocytes and macrophages was studied *in vitro*. Antigens prepared from *Dermatophagoides farinae* bodies (Dfb) or recombinant Mag3, glutathione-S transferase (GST)-fused mite antigen, stimulated murine spleen cells to proliferate. The responder cells were B cells, because the response was sensitive to anti-Ig antibody and C treatment, but not to anti-Thy 1 antibody and C treatment. The response was not due to lipopolysaccharide contamination, a representative B cell mitogen, because polymyxin B column-passed Dfb significantly stimulated B cells, and GST protein alone did not stimulate them. Alloantigen presenting activity was increased in mite antigen-treated B cells and spleen adherent cells. Mite antigens stimulated CD80 and the major histocompatibility complex (MHC) class II molecule expression, but suppressed CD86 expression on B cells and spleen adherent cells that were detected by a flow cytometer. Antibodies to the MHC class II molecules, CD80 and CD86 blocked the alloantigen-presenting activity. Furthermore, mite antigens stimulated B cells and spleen adherent cells to produce cytokines. These results suggest that mite antigens have a stimulating activity on antigen-presenting cells/macrophages and modulate immune responses.

Key words: mite antigen, antigen-presenting cell, cytokine, costimulatory molecule.

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Introduction

Allergens from house dust mites, *Dermatophagoides*, are major agents causing allergic disorders such as bronchial asthma, atopic dermatitis and perennial rhinitis [1]. The characterization of mite antigens has been made by biochemical, immunological and genetic engineering methods [2, 3]. Recently, cDNAs coding for major and important mite antigens have been cloned and the corresponding recombinant antigens have become available. The human and murine T cell-reactive region on Der p1 and Der p2 have been identified

using recombinant truncated fusion proteins, synthetic peptide, and/or T cell clones. Dr. Ono *et al.* have cloned four cDNAs coding for additional important allergens from *D. farinae* library which were referred to as *mite antigen gene* (*mag*) 1, 3, 29 and 44 [4–8]. The recombinant antigens, Mag1, Mag3 and Mag44, bound with considerably high frequencies to IgE antibody in the sera from patients with bronchial asthma and showing positive skin reactions to mite and house dust extracts, while Mag29 reacted to IgE antibody to a less extent [5]. Recombinant Mag1 and Mag3 stimulated T cells from mite-sensitive patients to proliferate [9, 10]. However, their biological activities were not fully analyzed. Previously, we reported that crude mite antigens, Dfb and Dff, have a potent B cell mitogenic activity in mice [11]. In this study, we investigated the effect of mite antigens on antigen presenting cells/macrophages from the standpoint of the production of cytokines and the expression of costimulatory molecules.

Materials and Methods

Mice

C3H/HeN and BALB/c mice were obtained from Seac Yoshitomi Experimental Animal Co. (Yoshitomi, Oita, Japan) and maintained on purina rodent chow and water *ad libitum*. Male mice were used at 8–12 weeks of age.

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 saturated NaCl solution and centrifuged at 1400×g for 30 min. The floating mites were collected by filtration and ground with quartz in 10 mM phosphate-buffered saline, pH 7.2 (PBS) and then centrifuged. The solubilized fraction was used as a body antigen. The antigen fraction was dialyzed and lyophilized. The freeze-dried extracts were referred to as Dfb. To exclude the contamination of lipopolysaccharide (LPS) in Dfb, Dfb was passed through a polymyxin B-Sepharose column [12].

Recombinant antigen was purified as described previously [8]. Briefly, an expression plasmid carrying *mag3* 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-Mag3 (66 kD) protein was obtained by passing it through a glutathione-Sepharose 4B (Pharmacia Biotech) column or by preparative disk gel electrophoresis (model NA-1800; Nippon Eido, Tokyo, Japan).

Preparation and fractionation of spleen cells

A spleen cell suspension was prepared from normal mice and treated with 0.83% NH₄Cl

in 0.017 M Tris buffer, pH 7.2, to lyse red blood cells. Four ml of spleen cell suspension (5×10^6 /ml) with RPMI 1640 (Nissui Seiyaku Co., Tokyo, Japan) containing 10% fetal calf serum (FCS, Grand Island Biological Co., Gland Island, NY, USA) was incubated in 60 mm plastic culture dishes (Falcon #3002, Becton Dickinson Co., Lincoln Park, NJ, USA) at 37°C for 2h in 5% CO₂ and 95% air. After incubation, nonadherent cells were discarded by gentle agitation with PBS. The adherent cells were obtained by scraping with a rubber policeman. The purity of adherent cells was <10% IgM⁺ cells and <5% Thy 1⁺ cells as determined by flow cytometry.

Purified B cells were prepared by passing spleen cells through a Sephadex G-10 column (Pharmacia Biotech) after treating the spleen cells with anti-Thy 1.2 antibody and rabbit complement (C, Cedarlane Laboratories, Ontario, Canada) at 37°C for 1h. The purity of B cells was >95%.

Purified T cells were prepared by passing spleen cells through a nylon column as described previously [13]. The purity of T cells was >95%.

Assay of proliferative response of spleen cells

Spleen cells (2×10^5) were cultured with mite antigens, Dfb (25 µg/ml), Mag3-GST (12.5 µg/ml), LPS (10 µg/ml, Sigma Chemical Co., St. Louis, MO, USA), or GST (12.5 µg/ml) in Enriched Hanks Amino Acid (EHAA) medium containing 10% FCS as described by Corradin *et al.* [14] in wells of flat-bottomed microtiter culture plates (Falcon #3072, Becton Dickinson Co.) at 37°C for 3 days in 5% CO₂ and 95% air. The cells were labeled during the last 18h with 18.5 kBq of tritiated thymidine ([³H]-TdR, specific activity, 222 Gbq/mmol, Amersham International plc, Buckinghamshire, UK) and were harvested using a semi-automated cell harvester (Abe Kagaku Co., Chiba, Japan). The amount of radioactivity incorporated into DNA in the lymphocytes was measured with a liquid scintillation spectrophotometer (Aloka, Tokyo, Japan) [15]. The results are expressed as the mean counts per minute (cpm) of [³H]-TdR incorporated by lymphocytes with standard error in triplicate cultures.

Allogeneic mixed lymphocyte reaction

Spleen adherent cells from C3H/HeN(H-2^k) mice were cultured with mite antigens at 37°C for 18h, treated with 100 µg/ml of Mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan), washed, and then cultured (5×10^4) with 2×10^5 responder T cells from BALB/c(H-2^d) mice in EHAA medium containing 10% FCS in flat-bottomed microtiter culture plates at 37°C for 6 days in 5% CO₂ and 95% air. The cells were labeled and harvested as described above.

Flow cytometric analysis

Spleen adherent cells (1×10^6) were incubated on ice for 30 min with a saturating amount

of fluorescein conjugated anti-CD80(clone 1G10), CD86(clone GL1), I-A^k(clone 14V.18) and Mac-1 (clone BL-N/G1, Cosmo Bio Co., Tokyo, Japan). After incubation, cells were washed with PBS and analyzed by EPIX XL (Coulter Co., Healea, FL, USA). At least 10,000 cells were counted for each sample. The results were expressed as the percentage and the profile of fluorescence-positive cells.

Production and assay of cytokines

Spleen adherent cells and B cells (5×10^5 /ml) were cultured with mite antigens in RPMI 1640 medium containing 10% FCS in 24 well culture plates (Falcon #3047, Beckton Dickinson Co.) at 37°C for 20h, and the culture supernatants were harvested. Cytokines in the culture supernatant were assayed by enzyme-linked immunosorbent assay (ELISA) as described previously [16] using anti-mouse cytokine monoclonal antibodies, biotin-labeled anti-cytokine monoclonal antibodies and streptoavidin-conjugated alkaline phosphatase (Pharmingen, San Diego, CA, USA) and p-nitrophenylphosphate (Zymed Laboratory Inc., San Francisco, CA, USA) as a substrate. The results were expressed as mean ng of cytokines produced by 5×10^5 cells in duplicate cultures comparing with recombinant cytokines (Pharmingen) as the standard.

Analysis of cytokine mRNA expression

Purified B cells (10^6 /ml) were stimulated with LPS or mite antigens at 37°C for 6h. Expression of cytokine mRNA was detected by semi-quantitative RT-PCR. Total cellular RNA was extracted by an acid guanidinium thiocyanate-phenol-chloroform extraction procedure [17]. First strand cDNA was synthesized from 5 µg of the total RNA by SuperscriptTM II RNase H⁻ reverse transcriptase (Life Technologies Inc., Rockville, MD, USA) as instructed by the manufacturer using 0.25 µg of random primer (Life Technologies Inc.). One-tenth of the synthesized cDNA was amplified by PCR using 50 pmol sense and antisense primers with 1 unit of Taq polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) in a total volume of 50 µl. The reaction buffer consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM dNTP. PCR cycles were performed for 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for extension, and at the first cycle denaturation was run for 2 min at 94°C. The sequences of the sense and antisense primers of IL-1α, IL-6 and β-actin were (5'-ATGGCCAAAGTTCTTGACTTGTTT-3', 5'-CCTTCACCCAACACGGGCTGGTC-3'), (5'-GACAACCACGGCCTTCCCTA-3', 5'-GGTACTCCAGAAGACCAGAGGA-3') and (5'-GTGGCTCCGCTCTAGGCACCAA-3', 5'-CTCTTTGATGTCACGCACGATTTC-3'), respectively. The product size of IL-1α, IL-6 and β-actin was 625, 303 and 360 base pairs, respectively. The PCR cycle of IL-1α was 38, IL-6, 29 and β-actin, 28. A 10 µl of PCR products was electrophoresed using a 1.5% agarose gel. After ethidium bromide staining (Sigma Chemical Co.), PCR products were visualized by UV illumination.

Statistical analysis

All experiments were repeated at least three times, and one representative result is shown in Tables and Figures. Statistical analysis was performed by the Student's *t* test. A confidence level of < 0.05 was considered significant [18].

Results

Mitogenic activity of mite antigens on spleen cells

At first, we studied the effect of mite antigens on the proliferative response of spleen cells. Crude mite antigen, Dfb, and recombinant antigen, Mag3-GST, and LPS, as a control mitogen, significantly stimulated normal spleen cells to proliferate at the concentration of 25 $\mu\text{g}/\text{ml}$, 12.5 $\mu\text{g}/\text{ml}$, and 10 $\mu\text{g}/\text{ml}$, respectively (Fig. 1). In order to exclude the contamination of LPS in the antigen fraction, we passed Dfb through a polymyxin B-Sepharose column (Dfb-PMB-S). This Dfb still retained its stimulating activity. On the other hand, the LPS response was markedly reduced by PMB-S column passing. Furthermore, GST protein

Fig. 1. Stimulating activity of mite antigens on murine spleen cells. Spleen cells (2×10^5) were cultured with 25 $\mu\text{g}/\text{ml}$ Dfb, PMB-S passed Dfb, 12.5 $\mu\text{g}/\text{ml}$ Mag 3-GST, 12.5 $\mu\text{g}/\text{ml}$ GST, 10 $\mu\text{g}/\text{ml}$ LPS or PMB-S-passed LPS at 37°C for 3 days. The cells were pulsed with 18.5 kBq of $[^3\text{H}]\text{-TdR}$ for the last 18h and harvested. $[^3\text{H}]\text{-TdR}$ incorporated by spleen cells was counted by a liquid scintillation spectrophotometer. The results are expressed as the means $\pm$ SE of cpm of $[^3\text{H}]\text{-TdR}$ incorporated by spleen cells in triplicate cultures.
*significantly stimulated.

Fig. 2. Responder cells are B cells for the stimulation with Dfb and Mag3. Spleen cells (2×10^5) treated with C alone (□), anti-Thy-1 antibody and C (▨) or anti-Ig antibody and C (■) were cultured with 10 μ g/ml LPS, 10 μ g/ml Con A, 25 μ g/ml Dfb or 12.5 μ g/ml Mag3-GST, for 3 days, and [3 H]-TdR incorporation was examined.

*significantly reduced from the group of C alone.

alone as the control of Mag3-GST had no stimulating activity. To determine the responder cells to mite antigens, we fractionated spleen cells using anti-Thy 1 or anti-Ig antibody and C treatment. As shown in Fig. 2, control cells responded well to Con A, T cell mitogen, LPS, B cell mitogen, and mite antigens, Dfb and Mag3. Anti-Thy 1 antibody and C treatment reduced the Con A response, but not the LPS response. Anti-Ig antibody and C treatment reduced the LPS response, but not the Con A response. The mite antigen responses were reduced by anti-Ig and C treatment, but not by anti-Thy 1 antibody and C treatment. These results suggest that the major responder cells for mite antigens are B cells, but not T cells. Dfb and Mag3 also stimulated Ig production by B cells (data not shown).

Alloantigen presenting activity of B cells and spleen adherent cells treated with mite antigens

Next, we studied the antigen presenting activity of B cells, another function of B cells. B cells from C3H/He mice were cultured with mite antigens at 37°C overnight, and then the alloantigen presenting activity to T cells from BALB/c mice was studied. Dfb and Mag3-treatment enhanced the alloantigen presenting activity of B cells (Table 1). A similar result was obtained using spleen adherent cells as another source of antigen presenting cells.

Table 1. Effect of mite antigens on alloantigen-presenting activity of spleen adherent cells

Antigen-presenting cells stimulated with	[³ H]-TdR uptake by T cells (cpm)	
	B cells	Spleen adherent cells
PBS	2766 ± 845	327 ± 43
LPS	3090 ± 415	5440 ± 486*
Dfb	7937 ± 278*	3151 ± 810*
Mag3	4646 ± 241*	1015 ± 174*
GST	3356 ± 87	247 ± 31

B cells and spleen adherent cells from C3H/HeN mice were incubated with 10 µg/ml LPS, 25 µg/ml Dfb, 12.5 µg/ml Mag3-GST or 12.5 µg/ml GST for 18h, then treated with 50 µg/ml mitomycin C for 1h. After washing, 5×10^4 cells were mixed with 2×10^5 T cells from BALB/c mice, cultured for 6 days, and [³H]-TdR incorporated by T cells was counted. The results are expressed as cpm of [³H]-TdR incorporated by T cells and SE in triplicate cultures. *significantly enhanced from the group of PBS.

These alloantigen presenting activities were blocked with anti-I-A^k, anti-CD80 and anti-CD86 antibodies (Fig. 3), suggesting that the antigen-presenting activity is mediated by these molecules.

Effect of mite antigens on cytokine production by B cells and spleen adherent cells

Antigen-presenting activity is mediated by cytokines and adhesion molecules. Therefore, we studied the effect of mite antigens on cytokine production. Purified B cells and spleen adherent cells were stimulated with mite antigens for 18h, and the production of IL-1α, IL-6, IL-10 and IL-12 in the culture supernatant was assayed by ELISA. Dfb and Mag3 markedly induced these cytokines on spleen adherent cells, while control protein, GST did not (Table 2). Purified B cells produced a small amount of IL-10, but did not produce any other cytokines (Table 2). However, by RT-PCR, cytokines such as IL-1α and IL-6 were detected in B cells after the stimulation with Dfb and Mag3 (Fig. 4). We also confirmed that Dfb-PMB-S had a similar activity to Dfb (data not shown).

Effect of mite antigens on cell surface marker on B cells and spleen adherent cells

Following the culturing of spleen adherent cells with mite antigens for 18h at 37°C, the expression of CD80, CD86 and I-A^k on the spleen adherent cells was detected by a flowcytometer. As shown in Fig. 5, spleen adherent cells cultured with PBS expressed 42% CD80, 77% CD86 and 82% I-A^k. The culture of spleen adherent cells with Dfb enhanced the expression of CD80 and I-A^k, but reduced the expression of CD86. Similar results were obtained using purified B cells.

Fig. 3. Effects of anti-CD80, anti-CD86 or anti-I-A^k antibodies on allogeneic MLR. Spleen adherent cells from C3H/HeN mice were cultured with 25 μ g/ml Dfb, 12.5 μ g/ml Mag 3-GST or PBS for 18h, and treated with 50 μ g/ml mitomycin C for 1h. After washing, 5×10^4 cells were incubated with PBS (□), anti-CD 80 (▨), anti-CD-86 (▩) or anti-I-A^k (■) antibody (1 μ g/ml) for 30 min, mixed with 2×10^5 T cells from BALB/c mice, and cultured for 6 days, and [³H]-TdR incorporated by T cells was counted. The results are expressed as percentage of T cell response in the presence of antibody when assuming that the T cell response without antibody is 100%.

*significantly suppressed.

Table 2. Effect of mite antigens on cytokine production by spleen adherent cells or B cells

Stimulated with	Spleen adherent cells				B cells
	IL-1 α	IL-6	IL-10	IL-12	IL-10
PBS	3265	433	37	365	8
LPS	98565	108435	170	5516	94
Dfb	69046	22705	45	4044	33
Mag3	53791	8143	100	4213	28
GST	1702	418	60	141	17

Spleen adherent cells and B cells (5×10^5 /ml) were cultured with several stimulators for 18h, the culture supernatants were harvested, and cytokines in the culture supernatant were measured by ELISA. The results are expressed as mean pg cytokines produced by 5×10^5 cells in duplicate cultures. The production of IL-1 α , IL-6, IL-12 by B cells were not detected.

Fig. 4. Surface marker expression on spleen adherent cells. Spleen adherent cells were cultured with PBS (A), Dfb (B) or Mag3-GST (C) overnight, stained with fluorescein anti-CD80, CD86, I-A^k or Mac 1 antibody, and analyzed by a flowcytometer. The results are expressed as the profile of fluorescein positive cells. Shadows show negative staining. The numbers in the figures are %positive cells and mean fluorescence intensity.

Discussion

Dermatophagoides is one of the major allergens that induces allergic diseases in humans. Antigens prepared from *Dermatophagoides* have several immunological properties that react with serum antibody and stimulate T cells from allergic patients [1–3]. In our previous papers we reported that mite antigens had an activity not only to specifically stimulate T cells but also to nonspecifically stimulate B cells to proliferate and to produce immunoglobulin in mice [9, 10, 11]. In this study, we investigated the effect of mite antigens on antigen-presenting cells/macrophages from the standpoint of cytokine production and costimulatory molecule expression. In this experiment we used crude *Dermatophagoides* antigen (Dfb)

B cells stimulated with

Fig. 5. Cytokine mRNA expression in B cells stimulated with mite antigens. Purified B cells were stimulated with PBS, LPS, Dfb, Mag3-GST or GST for 6h. After mRNA was extracted, RT-PCR was performed, and PCR-products were electrophoresed. The results are expressed as ethidium bromide-staining bands of each cytokine.

and GST-fused recombinant antigen (Mag3) as antigens [7]. Dfb and Mag3 nonspecifically stimulated murine spleen cells to proliferate, confirming our previous report [10]. Responder cells to mite antigens were B cells, but not T cells, because the mite antigen response was sensitive to anti-Ig antibody and C treatment, but not to anti-Thy 1 antibody and C treatment. A representative B cell mitogen is LPS. However, we confirmed that the B cell stimulating activity of mite antigens was not due to the contamination of LPS, because polymyxin B-column passed Dfb still retained B cell stimulating activity, and GST alone without Mag3 similarly prepared with Mag3-GST did not stimulate B cells. Polymyxin B-column passed LPS markedly lost its B cell stimulating activity. Mite antigens stimulated B cells and spleen adherent cells to produce cytokines such as IL-1, IL-6, IL-10 and IL-12. It has been reported that in allergic patients T cells polarize to Th2 in which IL-4-production is increased and IFN- γ -production is decreased [19], and IL-12 production by monocytes, which is an important cytokine to induce Th1, is decreased in allergic patients [20]. Furthermore, the immunization of mice with Der p1 decreased IFN- γ -production and IgG2a

antibody titer, while it did not affect IL-4-production and Ig isotype switching [21]. However, we could not detect any polarization of cytokine production at the antigen presenting cells/macrophage level. Costimulatory molecules such as CD80 and CD86 play an important role in the antigen presentation [22, 23, 24]. Therefore, we studied the effect of mite antigens on the expression of costimulatory molecules on antigen presenting cells. Spleen adherent cells expressed 42% CD80, 78% CD86 and 81% MHC class II molecules on their surface. Mite antigens enhanced the expression of MHC class II molecules and CD80, but decreased that of CD86 on B cells and spleen adherent cells. In agreement with these results, B cells and spleen adherent cells treated with mite antigens more efficiently stimulated allogeneic T cells to proliferate (alloantigen presentation) and anti-CD80, CD86 and MHC class II antibodies inhibited their antigen presentation. Although the percentage of CD86 expression on adherent cells was decreased by mite antigen-treatment, the suppressive effect of anti-CD86 antibody on the alloantigen presentation was the same between mite antigen-treated and non-treated spleen adherent cells. This means that the reduced CD86 expression still has enough antigen-presenting activity. CD40 was not detected in our system (data not shown). In this experiment we used purified B cells and spleen adherent cells as antigen-presenting cells.

Spleen adherent cells contained about 80% MHC class II-positive cells and 35% Mac 1-positive cells and 10% surface Ig-positive cells (B cells). We did not further purify the spleen adherent cells, because the further purification procedure nonspecifically stimulated cytokine production. Mite antigens similarly affected alloantigen presentation and CD80/CD86 expression of purified B cells. However, purified B cells did not produce a large amount of cytokines when stimulated with mite antigens.

Recently it has been reported that mite antigens have cysteine protease activity which digests CD23 and CD25 and modulates immune responses [25, 26]. Furthermore, King *et al.* reported that mite antigens containing cysteine protease stimulated human airway epithelial cells to produce cytokines such as IL-6 and IL-8 [27]. The stimulation mechanism of antigen-presenting cells/macrophages with mite antigens, whether this is mediated by protease activity or by specific receptors on macrophages, will be the next subject of this study.

Antigen-presenting cells/macrophages are the initial cells that handle foreign substances. Accordingly, the evidence that mite antigens have an activity that stimulates antigen presenting cells/macrophages is an important finding when considering the mechanism of T cell and B cell activation with mite antigens.

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抗原提示細胞/マクロファージの機能に及ぼすダニ抗原の影響

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要 旨: マウスのリンパ球とマクロファージの機能に対するダニ抗原の影響を *in vitro* で解析した。コナヒョウヒダニ虫体から精製した抗原 (Dfb) と GST-融合リコンビナント Mag3 ダニ抗原蛋白は、マウスの脾細胞の増殖反応を刺激した。この反応は、抗 Ig 抗体と補体の処理で低下するが、抗 Thy-1 抗体と補体の処理を受けないことから、反応性細胞は B 細胞であると考えられた。ポリミキシン B カラムを通過した Dfb や、GST 蛋白のみではこの作用がないことから、ダニ抗原による刺激は代表的な B 細胞マイトーゲンである LPS の混在によるものではないと考えられた。ダニ抗原で処理した B 細胞と脾付着細胞はアロ抗原提示能が亢進していた。ダニ抗原の処理は、B 細胞、脾付着細胞上の MHC クラス II 分子と CD80 の発現を増強したが、CD86 の発現は低下させた。MHC クラス II 抗原、CD80、CD86 に対する抗体はアロ抗原提示能を阻害した。さらにダニ抗原は B 細胞と脾付着細胞のサイトカイン産生を増強した。これらの事実はダニ抗原が抗原提示細胞/マクロファージの機能を刺激し、免疫反応を修飾しうる能力があることを示している。

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