

[ORIGINAL]

DERMATOPHAGOIDES PTERONYSSINUS SPECIFIC IgM ANTIBODY AND
ITS COMPLEMENT ACTIVATION IN CHILDREN
WITH BRONCHIAL ASTHMA

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Western blotting analysis and ELISA were used to examine Dp specific IgM abs in the sera and sputa of bronchial asthmatic and non-atopic children. With Western blotting Dp specific IgM abs were detected binding diffusely to high molecular weight (>65 kD) components of Dp extracts and from the intensities of the staining of the sera the asthmatic children were divided into two groups: an intensive staining and a faint staining group. The non-atopic children showed still fainter staining than the latter group. ELISA titers of Dp specific IgM abs in the intensive staining group were significantly higher than in the faint staining group and the non-atopic children ($p<0.01$). Dp specific IgM abs in sputa were also detected more intensively in asthma than in non-atopy by Western blotting.

The hemolytic complement activities of the fresh sera were consumed when they were incubated with Dp extracts at 37°C. The degree of consumption was significantly higher in the intensive staining group than in the faint staining group and the non-atopic children ($p<0.05$). High molecular weight fractions of Dp extracts were responsible for this complement inactivation.

From these results we suspected that Dp specific IgM abs might react with high molecular weight components of Dp to induce complement activation by the classical pathway and might cause chronic inflammatory changes in the respiratory mucosa. This chronic inflammation might emphasize IgE mediated asthmatic reactions to cause late phase reactions or chronic asthma.

Key words: Dp specific IgM — ELISA — hemolytic complement activity — high molecular weight components of Dp extracts — western blotting analysis

INTRODUCTION

In children IgE mediated mast cell degranulation and its pharmacological mediators are thought to play a leading part in the mechanism that induces asthmatic reactions. However, recent studies have suggested that inflammation may play an important role in characteristic bronchial

hyperresponsiveness and symptoms of chronic asthma, and that the roles of inflammatory cells, mediators, and neuropeptides have been recognized. Inflammatory cells can be attracted by IgE mediated chemical mediators, and their chemical products can cause inflammatory changes in the respiratory airway¹⁾²⁾, although the precise mechanisms to induce inflammation are still unclear.

The complement system is one of the major immune systems and can induce inflammation efficiently. Its activation in vivo after bronchial provocation tests with allergenic extracts in asthmatic patients has been reported. The results are inconsistent: some authors demonstrated decreased C3 and increased C4³⁾ or increased C3, C4

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Abbreviations: Dp *Dermatophagoides pteronyssinus*; ELISA enzyme-linked immunosorbent assay; abs antibodies; PBS phosphate buffered saline

and CH_{50} ⁴⁾; others indicated no significant changes in C3 or the C3d/C3 ratio⁵⁾. On the other hand, it has been consistently demonstrated that allergenic extracts activate the complement system of fresh sera in vitro^{6)–8)}. The precise mechanism of this complement activation by allergenic extracts and its role in bronchial asthma remained obscure^{9)–11)}.

In this report we examined Dp specific IgM abs, strong complement activators, in sera and sputa by Western blotting analysis. Dp specific IgM abs in sera were also quantitated by ELISA and were compared with the consumption of hemolytic complement activities when incubated with Dp extracted at 37°C. Dp specific IgM abs were detected against high molecular weight components of Dp extracts, and the hemolytic complement activities of fresh sera in the intensive staining group were inactivated by Dp extracts significantly more than those of non-atopy. These results suggested the significance of Dp specific IgM abs for the induction of inflammation in bronchial asthma.

MATERIALS AND METHODS

Patient population

Asthmatic patients admitted to Minami Kyoto National Hospital for more than one year with high Dp specific IgE abs were studied. The criteria for non-atopy were defined by no family or past history of three major allergic disorders. There were no significant differences in the titers of CH_{50} between the asthmatic (32.3 ± 6.9) and non-atopic children (33.7 ± 6.8).

Preparation of Dp extracts

Mass culture was performed essentially as described by Miyamoto et al.¹³⁾ Dp was cultured in a medium containing dry yeast and fish powder at 37°C and 75% humidity. Purified mites were separated by floatation with saturated NaCl solution. Dp was homogenized with an ultrasonic homogenizer in PBS, pH 7.4 at 4°C. Following one hour centrifugation at 4°C (60,000 ×g), the supernatant was filtered through a 0.22 µm filter (Millipore, Co.). Gel filtration of these crude extracts was performed with 5 ml samples on a 400

ml column (height 80 cm, diameter 2.5 cm) of Sephacryl S-300.

Western blot analysis

Crude Dp extracts were analyzed in 10% SDS-PAGE under nonreducing conditions, and transferred to a nitrocellulose sheet by electrophoretic blotting¹⁴⁾. The nitrocellulose sheet was incubated with 1% BSA in PBS and then incubated with 2-fold diluted sera at 4°C overnight. Dp specific IgM abs were detected by incubating the sheet with peroxidase-conjugated goat anti-human IgM (TAGO, Inc.) for 3 hours at room temperature. 4-chloro-1-naphthol in 0.01 M Tris-HCl (pH 5.4) with 0.15 M NaCl was used for the substrate. The washing buffer was PBS containing 0.05% Tween-20. Dp specific IgG, IgE and IgA were also detected in the same way.

Measurement of Dp specific IgM abs by ELISA

Microtiter plates (Immulon II) were coated with Dp extract by incubating them with 100 µl of 0.2 µg/ml in PBS overnight. After blocking with BSA, sample sera were added to the wells, incubated for 4 hr at room temperature followed by incubation with peroxidase-conjugated goat anti-human IgM. One hundred µl of the substrate solution (4 mM H_2O_2 and 8 mM 2,2'-azino-di (3-ethylbenzothiazoline-6-sulfonic acid) in 0.01 M citrate buffer, 0.15 M NaCl pH 4.5) was used. After stopping the reaction with 0.02% NaN_3 solution, the optical density at 410 nm was measured in duplicate. Between each incubation, the wells were washed with PBS including 0.05% Tween 20. ELISA assay was calibrated by use of dilutions of a reference serum and the antibody concentrations were expressed in units.

Hemolytic complement assay

Hemolytic complement assay was performed essentially as described by Rapp et al.¹⁵⁾ Briefly, sheep red blood cells 1.5×10^8 /ml in VBS-G sensitized with IgM hemolysin (kindly provided by Dr. Borsos, LIB-NCI) were incubated with serum diluted in sequence at 37°C for 1 hr, and % hemolysis at 412 nm (y) was measured. Z number was calculated by $Z = -\ln(1-y)$ and the dilution rate of the serum for $Z=1$ was calculated.

RESULTS

Western blot analysis of sera and sputa

Representative results are shown in Fig. 1-A and 1-B. Dp specific IgM abs were detected binding diffusely to high molecular weight components of Dp extracts (>65 kD) in the sera of bronchial asthmatics (Fig. 1). In contrast, no distinct binding was observed in non-atopic sera.

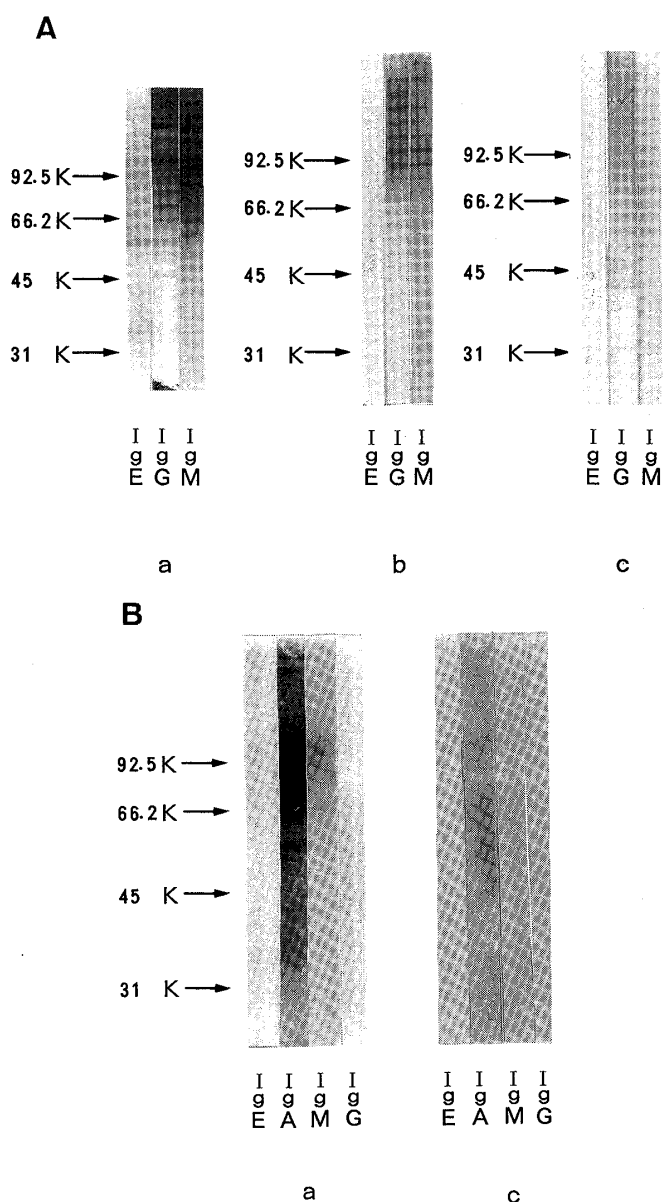


Fig. 1. Representative results of the Western blotting analysis of Dp specific IgE, IgG, IgM abs: a, intensive staining; b, faint staining; and c, non-atopy. Peroxydase conjugated antibodies were used diluted 500-, 250-, and 1000-fold for anti-IgE, IgG and IgM, respectively. (A: serum, B: sputa)

From the results of the Western blotting of the sera, the asthmatic children were divided into two groups: an intensive staining and a faint staining group. IgG abs also reacted diffusely with high molecular weight components of Dp extracts like IgM. In sputa Dp specific IgM and IgA abs were also detected and the stainings were more intensive in asthma patients than in non-atopic children. IgE and IgG abs were hardly detected in sputa.

Dp specific IgM abs in ELISA

Fig. 2 shows the Dp specific IgM titers in the non-atopy, faint staining, intensive staining without clear bands and the intensive staining with clear bands groups. Between the non-atopy and faint staining groups there were no significant differences but in both intensive staining groups significantly higher IgM titers were detected than in non-atopy ($p < 0.01$).

Consumption of hemolytic complement activity in fresh sera incubated with Dp extracts

The percentage consumption of the classical

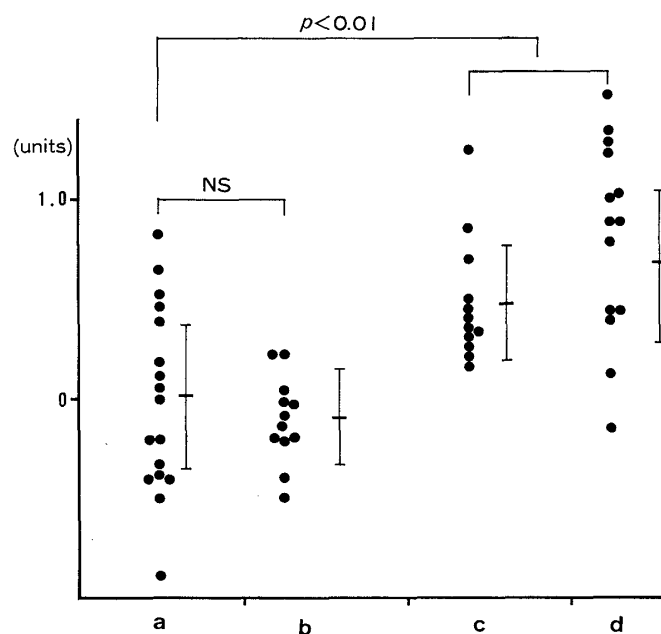


Fig. 2. Comparison of Dp specific IgM abs titers in ELISA between four groups; a, non-atopy; b, faint staining; c, intensive staining without clear bands and d, intensive staining with clear bands groups. Significant difference was observed between the non-atopy and both intensive staining groups ($p < 0.01$).

complement activity of fresh sera when incubated with Dp extracts (1 mg/ml) at 37°C for 1 hr was examined in the non-atopy, faint staining and

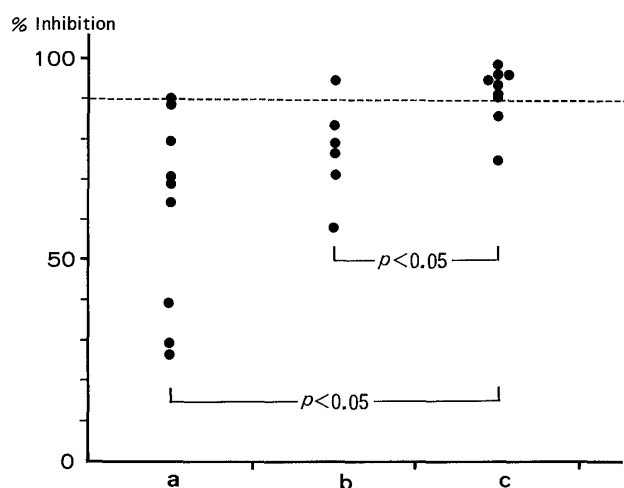


Fig. 3. Percentage inhibition of hemolytic complement activity when fresh serum was incubated with Dp extracts in the three groups: a, non-atopy; b, faint staining; and c, intensive staining groups. In asthmatic patients relatively higher complement consumption by Dp extracts was observed, and in the intensive staining group significantly higher complement inactivation was observed than in the non-atopic children and in faint staining group ($p < 0.05$).

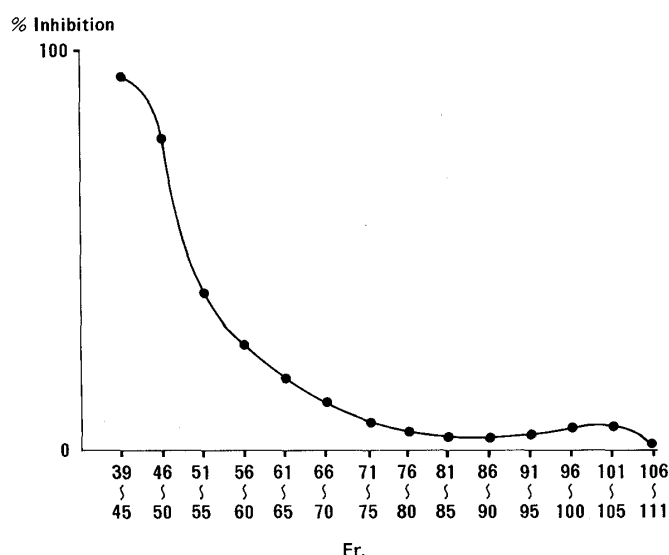


Fig. 4. Percentage inhibition of hemolytic complement activity when incubated with Dp fractions of the Sephacryl S-300 chromatography. Serum of the intensive staining group was incubated with equal volume of each fraction at 37°C for 1 hr and the hemolytic complement activity was examined.

intensive staining groups (Fig. 3). The optimal concentration of Dp extracts was determined to be 1 mg/ml in the preliminary study. In the intensive staining group, when the fresh serum was incubated with Dp, significantly higher complement consumption was detected than in the faint staining and non-atopy group ($p < 0.05$). In the cord serum without Dp specific IgM abs, low complement consumption (47.4 ± 10.1) was also detected. In order to examine the components of Dp extracts that caused this complement activation, Dp extracts in 0.9% NaCl were applied to a column of Sephacryl S-300 (2.5×80 cm). The eluates were incubated with the fresh serum at 37°C and hemolytic complement activity was examined. Fig. 4 reveals that the fractions which contained high molecular weight components of Dp extract significantly reduced hemolytic complement activity in serum. And fractions 66-75, which contained major Dp allergens (Der pI), hardly reduced hemolytic complement activity in serum at all.

DISCUSSION

In this report Dp specific IgM abs were examined by Western blotting and ELISA. In Western blotting asthmatic children showed distinct stainings, and these IgM abs were shown to bind mainly to high molecular weight components of Dp extracts. In contrast, non-atopic children showed only faint stainings. According to the intensity of the stainings, the asthmatic children were divided into two groups: a faint staining and an intensive staining group. In ELISA significantly higher IgM titers were detected in the intensive staining group than in the non-atopy and faint staining groups. Complement consumption by Dp extracts in fresh sera was also significantly higher in the intensive staining group than in the non-atopy or faint staining groups.

Aalberse et al.¹¹⁾ reported that activation of the classical pathway of human complement in vitro by house dust extracts was caused by IgM abs to polysaccharide antigen(s) derived from bacteria and they did not find any significant difference in complement activation between atopy and non-atopy. They used house dust extracts as allergens and compared adult patients with hay fever or

perennial rhinitis with adult non-allergic control. In this study we used purified mites as a source of Dp extracts and compared asthmatic children having high IgE abs to Dp with non-atopic children. In Western blotting IgM abs recognized high molecular weight components of Dp extracts (not low molecular weight components around 100–5000 D described as IgM binding components in house dust extracts by Aalberse et al.¹⁾. Between the faint and intensive staining groups, there was no significant difference in past histories of infectious diseases or in the duration of asthma (data not shown). So we suspect that the intensity of the binding of IgM might indicate increased sensitization to Dp and that this sensitization might be higher in asthmatic than in non-atopic patients. To establish the significance of IgM abs in bronchial asthma the determination of the precise components of Dp which were bound to IgM abs to activate the classical complement system, is necessary, however.

Past studies about complement activation in asthma revealed many conflicting results. Weemaes et al.⁸⁾ reported that hemolytic complement in the serum of almost every atopic child proved to be very sensitive to inactivation by purified house dust allergen. Berrens et al.¹⁸⁾ also indicated that activation by allergenic extracts is more readily achieved in the sera of allergic patients than in the sera of normal controls. Recently Michel et al.⁵⁾ failed to demonstrate complement activation during bronchial challenge test. But in vivo study it is difficult to evaluate the results because the changes in the complements in sera can not reflect the local complement activation in the respiratory mucosa. In our results complement consumption by Dp extracts with the optimal concentration of 1 mg/ml was observed in every sera, and the intensive staining asthmatic patient group showed greater consumption than the non-atopy and faint staining groups. The basal titers of CH₅₀ were not different in each group. The major part of the consumption of complement activity in non-atopy was considered to be unrelated to IgM abs because the complement activities of fresh cord sera were also consumed by Dp extracts, though the degree was lower than in non-atopy. The mechanism of this IgM non-related complement activation by Dp

extracts are now under study including the alternative pathway.

It was not surprising that in asthmatic sputa Dp specific IgA abs were detected but IgE and IgG abs were hardly detected at all. Dp specific IgM abs were also detected binding diffusely to the high molecular weight components of Dp extracts like in sera. Thomas A.E. et al.¹⁷⁾ claimed that natural exposure to mite allergens represents a very limited number of particles, each of which carries a much larger quantity of allergen than the droplets used for provocation. In this point Dp specific IgM abs in asthma could react with such Dp allergens in the respiratory mucosa to induce complement activation and chronic inflammations. This hypothesis might be consistent with the pathohistological results on necropsy, which showed complement (C3) in 3 and IgM in 11 of 18 asthmatic patients¹⁸⁾.

In summary we concluded that the intense binding of IgM abs to the high molecular weight components of Dp extracts could reflect the increased sensitization of asthmatic children to Dp and that Dp specific IgM abs and its classical complement activation in respiratory mucosa could be an additional important mechanism of bronchial asthma, especially of late asthmatic reactions or perennial asthma.

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気管支喘息児における、ヤケヒョウヒダニ特異 IgM 抗体と

その補体活性化能について

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ヤケヒョウヒダニ特異 IgM 抗体の存在を、Western blot 及び ELISA 両法にて、気管支喘息と非アトピー児について調べ、classical pathway を介する補体活性化能につき検討した。

Western blot 法によると同 IgM 抗体は、主にダニの 65K 以上の高分子領域と、び慢性に反応し、非アトピー児では弱い反応しか認めなかった。この結果、喘息群は intensive staining 及び faint staining の 2 群に大別された。

IgM ELISA 活性は intensive staining 群において有意に高値であった ($p < 0.01$)。ダニ抗原による血清中溶血性補体活性の吸収の程度も、intensive staining 群に有意に高かった ($p < 0.05$)。この吸収はダニ抗原の主に高分子成分との反応に基づくことが判明した。

以上、ダニ特異 IgM 抗体とダニ抗原高分子成分の反応に基づく classical pathway を介する補体の活性化が気管支喘息において一つの重要な役割を果たしていることが推察された。