

# Monoclonal antibodies to *Lepidoglyphus destructor*: delineation of crossreactivity between storage mites and house dust mites

B. HÄRFAST\*, M. VAN HAGE-HAMSTEN\*, I.J. ANSOTEGUI\*†, E. JOHANSSON\*, M. JEDDI-TEHRANI† and S.G.O. JOHANSSON\*

Departments of \*Clinical Immunology and †Immunology, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden

## Summary

We have developed monoclonal antibodies (MoAbs) to the storage mite *Lepidoglyphus destructor* (Ld). Employing these anti-Ld MoAbs (Ld-MoAbs) in ELISA and ELISA inhibition techniques we have analysed the reaction pattern of Ld-MoAbs to both non-pyroglyphid and pyroglyphid mites. The storage mite *Glycyphagus domesticus* (Gd) exhibited most efficient inhibition, followed by *Acarus siro* (As), *Tyrophagus putrescentiae* (Tp), *Dermatophagoides pteronyssinus* (Dpt) and *Euroglyphus maynei* (Em). Of the two pyroglyphid species, Dpt showed at least 1000 times less inhibition than Gd. Two of the MoAbs immunoprecipitated a band of 39 kDa whereas the third reacted weakly, with a high-molecular band of approximately 110 kDa. The Ld extract was also subjected to various reagents and conditions and the antigen was heat stable, it was not affected by low pH, or sensitive to dimethyl sulphoxide (DMSO) or paraformaldehyde. After exposure of the extract to various reagents, such as protease trypsin and periodate, we conclude that the epitopes recognized by Ld-MoAbs were of carbohydrate rather than of protein nature. It would thus seem that MoAbs recognize the carbohydrate part of a glycoprotein.

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## Introduction

Mites are considered to be one of the major causes of allergies in many parts of the world. House dust mites, belonging to the genus *Dermatophagoides*, have been extensively studied [1–4]. Two major allergens, a 25 kDa group I and a 14 kDa group II, have been identified, cloned and sequenced [5–7]. For several years we have concentrated on the much less studied storage mite *Lepidoglyphus destructor* (Ld). These mites are predominantly found in storage barns in farming environments where they feed on straw and grain dust. Farm workers exposed to barn dust are known to develop allergic reactions such as asthma, cough and rhinoconjunctivitis

[8–13]. Among Swedish farmers, we found mites to be the most important allergen, by far exceeding the allergic symptoms caused by pollen or animal danders [14]. We have previously described the allergen of Ld using sera from mite-allergic farmers and an immunoblotting technique [15]. Further, MoAbs have recently been developed by us [16] and others [17] to identify allergens in Ld.

The relation between storage mites and house dust mites as to allergenic crossreactivity has been addressed in several studies, using RAST and RAST-inhibition tests [18–20]. Naturally, as each serum contains its specific sets of polyclonal IgE antibodies, the pattern of reactivity is complex. We thought that the use of MoAbs might add some information as to the degree of crossreactivity to defined allergens between different mite species. In the present investigation we have used a set of MoAbs to Ld in an attempt to delineate crossreactivity between Ld and five other mite species.

Correspondence: Dr B. Härfast, Department of Clinical Immunology, Karolinska Hospital, S-10401 Stockholm, Sweden.

## Material and methods

### Mite extracts

*Lepidoglyphus destructor* (Ld), *Acarus siro* (As), *Tyrophagus putrescentiae* (Tp), *Glycyphagus domesticus* (Gd), *Dermatophagoides pteronyssinus* (Dpt) and *Euroglyphus maynei* (Em) were prepared from raw material obtained from Allergon Ab (Ängelholm, Sweden). Extracts were prepared in 0.15 M NaCl as 1/20 (w/v) for 18 hr at room temperature. The extracts were then centrifuged at 6600 g for 10 min and filtered through a 0.8 µm Millex-HA filter (Millipore, Bedford, U.S.A.). The protein concentrations of these extract were determined to be around 2 mg/ml. For some purposes, extracts were desalted on a PD10 Sephadex column (Pharmacia, Uppsala, Sweden) and subsequently lyophilized. All extracts were stored at -20°C until used.

### Monoclonal antibodies

BALB/c mice, aged 6–8 weeks, were immunized three times intraperitoneally with Ld extract. For the first injection, Ld was mixed with Freund's complete adjuvant whereas in the following two, Freund's incomplete adjuvant was used. The injections were given fortnightly. Hybridomas were produced according to standard procedures [21]. Briefly, 3 days after the final injection the mice were killed, spleen cells were mixed with SP2/0 myeloma cells and fusion was performed in a suspension of 50% polyethylene glycol 4000 (gas chromatography pure, Merc, Darmstadt, Germany). Cells were subsequently resuspended in a selective medium containing hypoxanthine, aminopterin, thymidine and seeded to microtitre plates containing peritoneal macrophages as feeder cells.

For assessment of antibody activity, culture supernatants were tested against Ld extracts using an enzyme-linked immunosorbent assay (ELISA) [22]. These assays were performed in microtitre plates (Nunc, Roskilde, Denmark). Briefly, wells were coated with 200 µl of a solution of Ld extract (1 µg/ml) in 0.05 M carbonate buffer, pH 9.6, overnight at room temperature. Phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin and 0.1% Tween 20 was used as a diluent. PBS containing 0.05% Tween 20 was used for washing throughout. First, the culture supernatants to be tested were diluted, added to wells and incubated for 4 hr. Next, 100 µl alkaline-phosphatase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts A/S, Copenhagen, Denmark) diluted 1/100 was added and incubated overnight. Finally, the substrate *p*-nitrophenyl phosphatase disodium (Sigma, St Louis, Missouri, U.S.A.) was diluted in 1 M diethanolamine buffer, pH 9.8, and added.

The absorbance at 405 nm was measured 30–60 min later with an ELISA plate reader. Clones displaying antibody activity were further cultivated, cloned and retested in ELISA against several other mite extracts. MoAbs from selected clones were subsequently isotyped and subclass determined using ELISA (and reagents from Southern Biotechnology Associates, Birmingham, Alabama, U.S.A.). In this study we selected three MoAbs for further analyses. One was of IgG3 subclass (42B6) and two were IgM (83F3 and 814C7). MoAbs of IgG isotype were in some instances purified from cell culture supernatant using protein G-Sepharose according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). Unless otherwise stated, cell culture supernatants were used as the source of MoAbs.

### Treatment of Ld extract

In an attempt to further characterize the nature of antigen recognized by the MoAbs, we treated the Ld extract in various ways. ELISA plates were coated with Ld as described above and were subsequently subjected to one of the treatments listed below. However, boiling of Ld extract was performed prior to coating of the ELISA plates. The following treatments of Ld extracts were performed: 90% dimethyl sulphoxide (DMSO), 80% methanol, 4% paraformaldehyde, 1% glutaraldehyde, 3% 2-mercaptoethanol (2ME), 2% sodium dodecylsulphate (SDS), citric buffer, pH3 (these were all treated for 1 hr at room temperature); boiling (5–30 min), 0.1 M sodium metaperiodate (periodate), for 3 days at 4°C in the dark; 0.5% trypsin 37°C, protease 250 µg/ml 37°C, *N*-Glycosidase F 18 units/ml (Boehringer, Mannheim, Germany), *O*-Glycosidase 20 units/ml (Boehringer). The enzyme treatments were all performed at 37°C for 18 hr. To test for the effects of these treatments, the ELISA activity of MoAb 42B6 to treated and untreated antigen was compared.

### ELISA inhibition assay

To study the specificity of the Ld-MoAbs and to assess the distribution of the antigen detected, we performed inhibition experiments. Ld-MoAbs diluted 1:200 were preincubated with six different mite extracts. In each experiment 12 different concentrations of each extract were tested. Starting with a 1:30 dilution, each extract was successively diluted using three-fold steps. Following a 2 hr incubation, the mixtures were transferred to microtitre plates coated with Ld and allowed to incubate for an additional 3 hr, followed by over-night incubation with rabbit anti-mouse immunoglobulin conjugate. This approach allowed us to determine the dilution of each extract which gave a 50% inhibition.

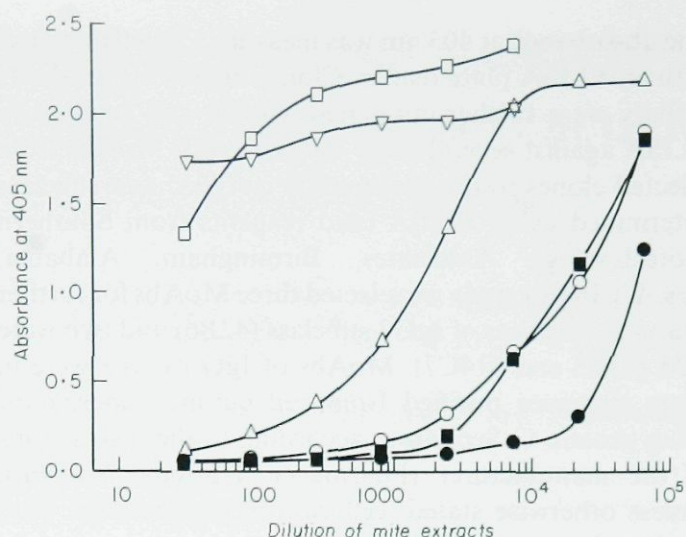


**Fig. 1.** The ability of six different mite extracts to inhibit the reaction of MoAb 42B6 to Ld in ELISA. ■, As. △, Tp. ▽, Em. □, Dpt. ○, Ld. ●, Gd.

#### *Radiolabelling of Lepidoglyphus destructor proteins*

One milligram of Ld extract was iodinated with 1 mCi of sodium  $^{125}$ iodine, using the commercial solid phase radioiodination reagent 1,3,4,6-tetrachloro-3a, 6a-diphenyl glycouril (Iodo-Gen) (Pierce Chemical Company, Rockford, U.S.A.) according to the manufacturer's instruction.

#### *Immunoprecipitation and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE)*

The anti-Ld MoAbs and immunoprecipitation control MoAb (1B3, a MoAb to p56<sup>lck</sup>) [23] were incubated with 100  $\mu$ l of protein-A Sepharose coated with goat anti-mouse immunoglobulins. After 1 hr, the antibody-bound protein A-Sepharose beads were washed three times with PBS. Samples were then incubated for 1 hr under continuous mixing with  $10 \times 10^6$  c.p.m. of preabsorbed radiolabelled Ld extract. The immunocomplex-bound protein A-Sepharose beads were washed six times with PBS, and then 60  $\mu$ l of sample buffer was added to these beads. After boiling for 3 min, the protein-containing sample buffers were analysed using 10% SDS-PAGE. Pre-stained BioRad (BioRad, Richmond, California, U.S.A.) molecular weight markers were used as SDS-PAGE standards. Gels were dried and autoradiographed on Amersham film with Agfa-60 intensifying screens (Agfa-Gevaert, Germany) at  $-70^\circ\text{C}$ .

## **Results**

### *Specificity*

We selected three MoAbs 42B6, 82F3, 814C7 for a thorough investigation of their reactions with Ld and five

other mite extracts. In a set of inhibition experiments the pattern of crossreactivity was investigated (Figs 1–3). In this assay, MoAbs 42B6, 82F3 and 814C7 were preincubated with serial dilutions of the panel of mite extracts. After preincubation, the antibodies were assessed against Ld in ELISA. The four storage mite extracts exhibited very strong inhibition. The most effective inhibitory storage mite extracts were, in order of efficiency; Gd, Ld, As, Tp. The two house dust mite species were ineffective by comparison (Figs 1–3). The six mite species may be divided into three groups according to their inhibitory capabilities (Table 1). The two Glycyphagidae species were more efficient than the two Acaridae species which in turn were more efficient than the two Pyroglyphidae species. For inhibition, 1  $\mu\text{g}/\text{ml}$  of the extracts were used. The pattern of inhibition was similar whichever of the MoAbs was used.

### *Resistance of Ld extract*

To characterize further the antigen recognized by the MoAb, extracts were exposed to various treatments (Table 2). These antigen samples were subsequently used as coating antigens in ELISA and compared with untreated antigens. The epitope recognized by MoAb 42B6 was very resistant to protease, trypsin, boiling (5 min), SDS, glutaraldehyde, paraformaldehyde, DMSO, methanol, low pH (pH 3) and 2ME. However, periodate treatment and boiling for 30 min reduced the ELISA response considerably. Also, treatment with *N*-glycosidase F reduced the response by 29% whereas *O*-Glycosidase was ineffective.

To check the effect of some of these treatments we also used another MoAb we recently raised against Ld. This MoAb is directed to the 15 kD band as analysed in immunoblotting. We now used MoAb 12D8 as a control to ensure that periodate oxidation would not generally destroy protein structures of Ld under the conditions used. As can be seen in Table 3, MoAb 12D8 displayed reduced response when tested against protease-treated extract but was not influenced by periodate oxidation of Ld. In contrast, 42B6 had the reversed pattern of sensitivity for these treatments of Ld.

### *Immunoprecipitation*

Confirming our previous results [16], we found that 42B6 precipitated a band of 39 kD. Here MoAb 814C7 also reacted with the 39 kD component. In contrast, MoAb 82F3 did not react with 39 kD component. Instead it precipitated a faint high-molecular band of approximately 110 kD. Although this faint band was repeatedly found its significance remains to be proved. Despite this, 82F3 displayed high ELISA activity.

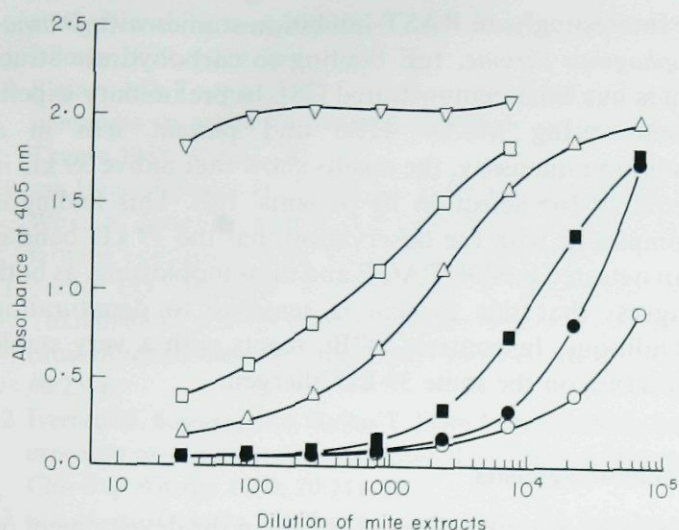


Fig. 2. The ability of six different mite extracts to inhibit the reaction of MoAb 82F3 to Ld in ELISA. ■, As. △, Tp. ▽, Em. □, Dpt. ○, Ld. ●, Gd.

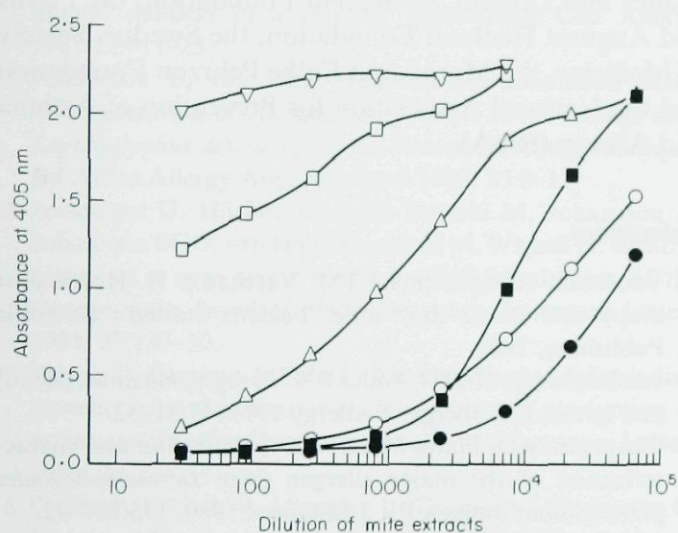


Fig. 3. The ability of six different mite extracts to inhibit the reaction of MoAb 814C7 to Ld in ELISA. ■, As. △, Tp. ▽, Em. □, Dpt. ○, Ld. ●, Gd.

Table 2. Influence of treatments of *Lepidoglyphus destructor* extract on the ELISA reaction of MoAb 42B6

| Exposure to      | % Diminished ELISA response |
|------------------|-----------------------------|
| Methanol         | 9                           |
| Paraformaldehyde | 0                           |
| Glutaraldehyde   | 0                           |
| SDS              | 0                           |
| DMSO             | 12                          |
| Mercaptoethanol  | 7                           |
| Protease         | 0                           |
| Trypsin          | 4                           |
| Periodate        | 87                          |
| N-Glycosidase F  | 29                          |
| O-Glycosidase    | 0                           |
| pH 3             | 4                           |
| Boiling 5 min    | 10                          |
| Boiling 30 min   | 84                          |

Table 3. Effect of treatment of *L. destructor* extract with periodate or protease on the ELISA results using MoAb 42B6 directed to 39 kD and MoAb 12D8 directed to 15 kD

| MoAbs | % Diminished ELISA response |          |
|-------|-----------------------------|----------|
|       | Treatments                  |          |
|       | Periodate                   | Protease |
| 42B6  | 76                          | 6        |
| 12D8  | 0                           | 81       |

Table 1. Comparison of the percentage inhibition obtained by different mite species on the ELISA response to *L. destructor*

| MoAbs | % Inhibition by mite extracts (1 µg/ml) |    |          |    |               |    |
|-------|-----------------------------------------|----|----------|----|---------------|----|
|       | Glycyphagidae                           |    | Acaridae |    | Pyroglyphidae |    |
|       | Ld                                      | Gd | As       | Tp | Dpt           | Em |
| 42B6  | 80                                      | 80 | 38       | 20 | 0             | 4  |
| 814C7 | 90                                      | 95 | 62       | 31 | 15            | 1  |
| 82F3  | 96                                      | 96 | 81       | 41 | 29            | 0  |

## Discussion

In the present study we describe a set of MoAbs raised against the storage mite Ld. These MoAbs were used as tools to investigate the relationship between Ld and five other mite species. In a number of previous investigations we [10,19,24,25] as well as other investigators [4,18,20] studied the clinical significance of allergenic crossreactivity between mites of various species. These experiments were performed using sera of mite-allergic patients. The specificity and pattern of reaction of IgE in these sera were assessed by means of RAST and RAST-inhibition studies. Although the results are not identical, a pattern emerged of a close relationship, within the storage mites and within house dust mites, respectively. Also, each species studied seemed to possess unique allergens in addition to allergens shared by closely related species. In

contrast, the crossreactivity between the two groups was limited, but not absent. For example, using an immunoblot technique we have recently shown that Ld shares some antigens with Dpt [26]. Such crossreactivity has also been suggested by others [18,20,27]. The present experiments using Ld-MoAbs are in line with the main finding that a closer taxonomical relationship infers more cross-reactivity. Thus the two house dust mite species displayed poor inhibition of the anti-Ld MoAbs, whereas within 'the storage-mite group', species belonging to the family of Glycyphagidae (Gd and Ld) were more efficient than species of the Acaridae family (As and Tp) in this respect. These results agree with our previous RAST inhibition data except for the reposition of Tp and As, i.e. Tp crossreacts more than As with Ld in experiments with sera from mite-allergic patients [24].

In agreement with previous characterization [16], we found that Ld-MoAbs recognized an antigen of 39 kD as determined by immunoprecipitation. In addition, we found a MoAb (82F3) that recognized a weak band of approximately 110 kD. This antibody seems to crossreact somewhat more strongly with Dpt than the other two Ld-MoAbs do. Whereas the 39 kD has been shown to be an allergen [16] this remains to be determined for the component recognized by 82F3. A series of experiments on the antigen recognized by 42B6 indicated that this was extremely resistant to a variety of treatments. Its resistance to trypsin and protease seems to suggest that the epitope recognized is not of protein nature but rather of carbohydrate. This is further supported by the sensitivity of Ld extract to periodate oxidation prior to ELISA analysed by 42B6. As control, in these experiments, we used a recently raised MoAb (12D8) directed to the 15 kD of Ld. In contrast to 42B6, the ELISA reaction was not influenced by periodate oxidation of Ld, whereas protease treatment decreased ELISA reactivity. This experiment implies that periodate oxidation does not generally under these conditions destroy the proteins of Ld. Moreover, treatment of Ld by *N*-Glycosidase F, an enzyme which will cleave intact oligosaccharides from glycoproteins, partly reduced the response of 42B6. The reduction was far from complete. However, according to the manufacturer's (Boehringer) information, complete digestion is not to be expected. It would thus seem that 42B6 recognizes the carbohydrate part of glycoproteins of 39 kD. Alternatively, the antigens could be pure carbohydrates of the same size. The latter possibility seems, however, to be excluded as the radiolabelling technique we used labels proteins only. Consequently, only antigens of protein nature could be visualized by immunoprecipitation. However, these antigens seem to be exclusively or preferentially expressed in the storage mite species.

Interestingly, in RAST-inhibition studies with *Dermatophagoides farinae*, IgE binding to carbohydrate structures has been demonstrated [28]. In preliminary experiments using MoAb 42B6 and patient sera in a radioimmunoassay, the results show that native 39 kD is required for detection by patients' IgE. This finding is compatible with the observation that the 39 kD band is not detected in SDS-PAGE and immunoblotting, as both suggest that this protein is sensitive to denaturing conditions. In contrast, 42B6 reacts with a very stable structure on the same 39 kD allergen.

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