

Detection of B-lymphocytes secreting antibodies to *Dermatophagoides* antigens

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Summary

An enzyme-linked immunospot assay (ELI-spot assay) has been established to count individual cells secreting antibodies to *Dermatophagoides* spp. allergens. Initial optimization of the assay was performed using *Der p* I-specific murine hybridoma cell lines. Inhibition with soluble purified allergen demonstrated that spot formation was specific. Addition of cycloheximide showed that spot formation was due to antibodies actively produced and secreted and not caused by preformed surface-associated Ig. An analogous method was used to count immunoglobulin-secreting cells (IgSC) of any specificity, and the number of circulating IgSC was measured in young healthy subjects. The results (means) were 331 IgM-SC, 532 IgG-SC, 850 IgA-SC and less than 1 IgE-SC per 10^6 mononuclear cells (MNC). Initial experiments performed with blood from patients undergoing immunotherapy with *Dermatophagoides pteronyssinus* Alutard, showed that 1 week after injection, circulating allergen-specific antibody-secreting cells (AbSC) were detected with this assay. The ranges were between 1 and 13 IgM-, 2 and 20 IgG- and 20 and 55 IgA allergen-specific AbSC per 10^6 MNC. It is expected that this assay will help to understand more thoroughly the basic immunological mechanisms behind the efficacy of immunotherapy.

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Introduction

Specific immunotherapy has often been used for patients with IgE-mediated allergy to inhalant allergens. Several studies have documented the clinical efficacy of this treatment [1–6]. In order to explain this, intensive research has concentrated upon the specific antibody response in serum in connection with immunotherapy (for review see [7]). Nevertheless, it has not been possible to demonstrate any definite correlation between the outcome of the treatment and the specific antibody response with respect to amount of class and subclass distribution. In order to obtain a better understanding of the basic mechanisms behind immunotherapy we have developed assays to quantify B-cells secreting antibodies to *Dermatophagoides pteronyssinus* allergens. It is known that the results obtained from measuring circulating specific B cells are different from the plasma antibody

response. It is therefore expected that the specific B-cell response will show a better correlation to the clinical efficacy of immunotherapy.

In this paper we present a simple, specific and sensitive method to count the individual cells secreting IgM, IgG, IgA and IgE antibodies to house dust mite antigens. In the so called enzyme-linked immunospot assay (ELI-spot assay) the principles of the traditional ELISA are used. Instead of measuring antibodies in the fluid phase, the individual specific antibody-secreting cells (AbSC) are detected and visualized as blue spots in the bottom of a microtitre well.

Materials and methods

Hybridoma cell lines

For initial optimizing of the ELI-spot assay the murine hybridoma cell line NHP4 was applied. The cell line secretes antibodies of the IgG₁ isotype, specific for the major allergen of *D. pteronyssinus*, *Der p* I [8,9].

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Subjects

Blood was obtained from eight young, healthy, adult volunteers. Blood for counts of *Dermatophagoides* AbSC was obtained from three patients undergoing immunotherapy with *D. pteronyssinus* Alutard (ALK, Denmark). Blood was taken on day 7 after injection.

Isolation of mononuclear cells

Mononuclear cells (MNC) were isolated from heparinized blood by Ficoll-Isopaque gradient (Nycomed, Oslo, Norway) soon after withdrawal. After centrifugation (400 g, 20°C, 30 min) the cells were collected and washed four times in 10 ml RPMI-1640 culture medium (Gibco, Herley, Denmark) (400 g, 20°C, 10 min). After the final wash the cells were resuspended in RPMI-1640 with 10% foetal calf serum (FCS) (SeroMed, Roskilde, Denmark).

Extracts

D. pteronyssinus extracts were produced from full grown whole mite cultures (WMC) obtained from ALK, Denmark. The cultures were extracted in ammonium hydrogen carbonate buffer pH 7.6 (1:10, w/v) at 5°C for 24 hr, followed by centrifugation (40 000 g, 4°C, 1 hr), dialysis (3 × 24 hr) and freeze-drying [10].

Der p I

Excrement fractions were isolated from WMC. *Der p I* was obtained by acetone extraction and precipitation of the excrement fractions, followed by hydrophobic interaction chromatography as described previously [11,12].

Counting allergen-specific antibody-secreting cells

The number of allergen-specific AbSC was measured by a modified ELI-spot method originally described by Czerkinsky *et al.* [13]. Microtitre plates (96-well, Maxi sorp, Nunc, Roskilde, Denmark) were coated overnight at 20°C with 50 µg/ml WMC extract in phosphate-buffered saline (PBS) pH 7.2, 100 µl in each well. After washing three times with PBS containing 0.05% Tween 20 (Merck, Darmstadt, FRG) and once with PBS, hybridoma cells or MNC were resuspended in RPMI-1640 medium with 10% FCS and applied in a volume of 100 µl (5×10^5 MNC/well). The cells were then incubated in a humidified atmosphere containing 5% CO₂ at 37°C for 150 min. Following incubation the plates were washed three times for 3 min with PBS containing 0.05% Tween 20 (Merck), 200 µl/well. After washing, peroxidase-conjugated rabbit antibodies to either human IgG, IgM, IgA and IgE or mouse Ig (Dako, Copenhagen, Denmark, P214, P215,

P216, P295, and P260 diluted 1:1000 (v/v) in PBS with 0.5% BSA) were added to detect bound antibody. After incubation at 20°C for 60 min the plates were washed three times and the bound antibodies were visualized by addition of an agarose substrate solution, 100 µl/well. The agarose substrate solution was prepared from the following stock solutions: (1) agarose stock solutions: 0.75% agarose in citrate phosphate buffer, pH 5, (Na₂HPO₄ (Merck) 14.39 g, citric acid (Merck) 10.36 g, distilled water to 1000 ml); (2) DONS stock solution: dioctyl sodium sulphosuccinate (Merck) 4 g, 96% ethanol to 500 ml; (3) TMB stock solution: 3,3',5,5'-tetramethyl benzidine 1.8 g, DMSO (Merck) to 25 ml. Finally, the agarose substrate solution was prepared from DONS stock solution (3 ml), TMB stock solution (100 µl), agarose stock solution (56°C, 12 ml), and H₂O₂ (6 µl). The substrate must be prepared immediately before use. After approximately 20 min incubation, blue spots appeared in the bottom of the well, each representing a specific AbSC. The spots were counted in a low magnification microscope with the microtitre plates illuminated from above.

Counting immunoglobulin-secreting cells (IgSC)

The allergen-specific assay was modified to measure all IgSC. Using the principles of a capture ELISA, the solid phase was coated with rabbit antibodies to either mouse Ig or human Ig (Dako, Z259 and A107 diluted 1:1000 (v/v) in PBS) instead of antigen. For the detection of IgE-SC a high affinity murine monoclonal antibody was applied. ELI-spot assays were performed as described above.

Cycloheximide inhibition

Spot inhibition was performed by the addition of 200 µg/ml cycloheximide (Sigma, Labkemi, Fredensborg, Denmark) to the cell incubation medium. The cells were incubated in a 5% CO₂ incubator at 37°C for 120 min before application in the allergen-coated wells. All other assay conditions were as described above.

Spot inhibition with specific allergen

Specific inhibition was performed by the addition of different concentrations of *Der p I* to the cell incubation medium. The most important allergen of birch, *Bet v I*, was applied as control allergen [14]. The other assay conditions were as described above.

Statistics

The results on human circulating IgM-, IgG- and IgA-SC were compared by means of a two-way analysis of variance.

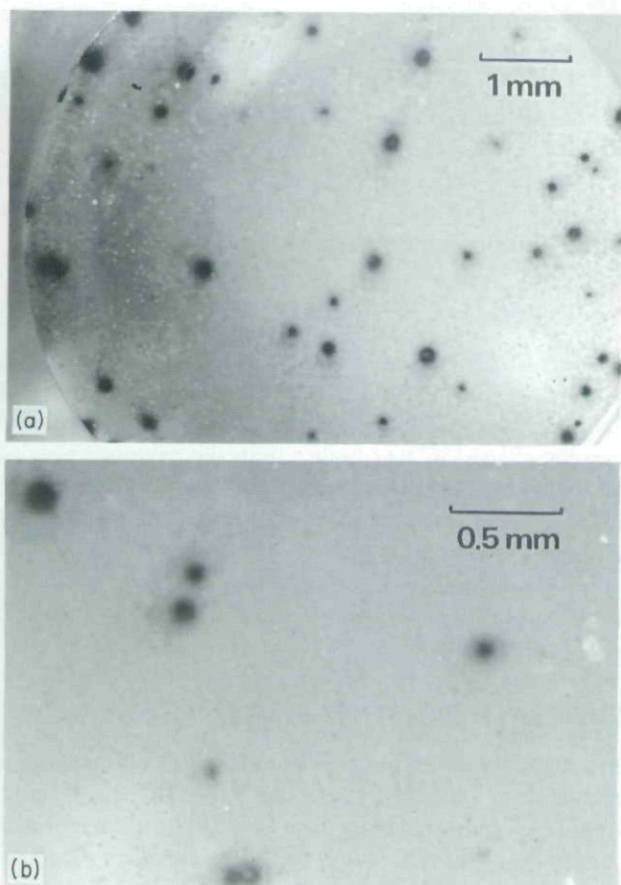


Fig 1. (a and b) The typical appearance of spots seen through a low magnification microscope (WILD M3Z).

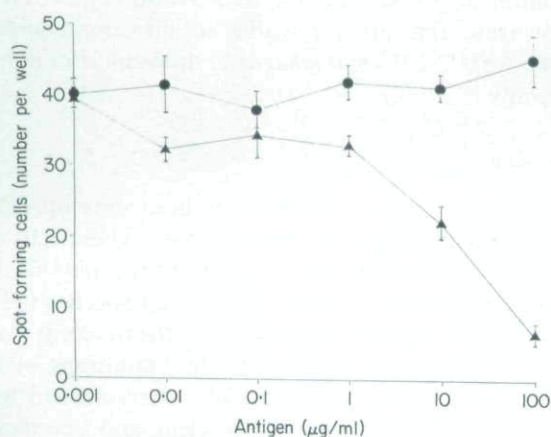


Fig. 2. Number of spot-forming cells per well after the addition of purified *Der p* I (▲) and *Bet v* I (●) with 95% confidence limits shown. The *Der p* I-specific murine hybridoma cell line NHP4 was incubated with different concentrations of antigen added to the cell incubation medium.

Table 1. The number of IgM-, IgG-, IgA, and IgE-SC/10⁶ MNC in young healthy persons ($n=8$)

	Range	Mean $\pm$ s.e.
IgM-SC	163–600	331 $\pm$ 60
IgG-SC	290–1018	532 $\pm$ 60
IgA-SC	550–1225	850 $\pm$ 60
IgE-SC	0–2	<1

Each measurement was performed in four replicates. A two-way analysis of variance was performed to describe the significances of IgM-, IgG- and IgA-SC.

Table 2. The number of *Dermatophagoides pteronyssinus* extract-specific spot-forming cells per 10⁶ MNC

	Patient no.		
	1	2	3
IgM-SC	1	13	3
IgG-SC	20	2	25
IgA-SC	55	20	33

Patient 1, 2 and 3 had been injected with 560, 1200 and 5300 SQ units of *Dermatophagoides pteronyssinus* Alutard, respectively. Blood was taken on day 7 after injection and each measurement was performed in duplicate in two dilutions.

The number of specific IgM-, IgG- and IgA-SC detected from each patient is shown.

Results

Several *Der p* I-specific murine hybridoma culture [8] supernatants were employed to establish optimal assay conditions, using a traditional ELISA [15]. Different coating buffers (pH ranging from 6 to 10) were assayed and PBS pH 7.2 was chosen on the basis of optimal absorption values. Experiments were performed to select the optimal coating time and 18 hr was chosen as the routine coating time.

Following these experiments the corresponding *Der p* I-specific hybridoma cell lines were used to optimize the *D. pteronyssinus*-specific ELI-spot assay. The wells were coated with different concentrations of WMC extract and on the basis of spot number and background, 50 µg/ml was chosen as optimal coating concentration. Optimal incubation time with cells was found to be 150 min.

In parallel with the coating conditions, different secondary antibodies, enzymes, incubation time were assayed, and the optimal reagents and parameters were chosen on the basis of spot number and background.

The hybridoma cell lines are well characterized with respect to specificity and affinity coefficient [9]. They could therefore be used to describe the sensitivity of the ELI-spot assay.

Figure 1 shows spots as they appear through a low magnification microscope. They exhibit the typical morphology of a spot: the colour is dense in the middle and becomes diffuse towards the periphery, illustrating that the AbSC is situated in the centre. Up to 200 spots can be counted in each well without problems of confluence of the spots (results not shown).

Simultaneously with the allergen-specific ELI-spot assay the principles of a capture ELISA were applied to set up a positive control assay to measure the number of IgSC among the hybridoma cells. As it was well known that all these hybridoma cells were *Der p* I-specific AbSC this assay could be used for comparative studies. A series of experiments was performed to elucidate whether the specific ELI-spot assay detected all the hybridoma IgSC. These results showed that the number of specific AbSC did not differ considerably from the number of IgSC (range 80–120%). However, the specific spots were generally slightly smaller, probably due to the difference in the affinities of the two assays.

Demonstration of spot specificity

The specificity of the allergen-specific ELI-spot assay was tested using the addition of allergen to the incubation medium to inhibit spot formation. As illustrated in Fig. 2 purified *Der p* I inhibited spot formation in a dose-dependent manner. The inhibition was statistically significant for concentrations of 1 µg/ml and above ($P < 0.05$). The remaining spots were smaller than those of the control, indicating a partial inhibition of spot formation, although this is not evident from the spot numbers. No inhibition was found when the control allergen, *Bet v* I, was added.

ELI-spot assay with human MNC

The initial work with human MNC involved establishing an ELI-spot assay to measure the number of circulating IgM-, IgG-, IgA- and IgE-SC. The principles of the ELI-spot assay measuring murine IgSC were transferred and modified to detect human IgSC among MNC isolated from peripheral blood.

Inhibition with cycloheximide

In order to exclude spot formation caused by membrane-bound immunoglobulin, cycloheximide was applied to inhibit spot formation by blocking protein synthesis. Cycloheximide (200 µg/ml) was added to the cell incuba-

tion medium 2 hr prior to application in the antibody-coated wells. The experiments were performed three times and IgSC were counted in four replicates with and without cycloheximide. The results showed a 94% inhibition of IgM-SC (range 82–97%), 97% inhibition of IgG-SC (range 97–98%), and 93% of IgA-SC (range 89–99%). This implies that spot formation was caused by cells that actively produce and secrete antibodies.

Immunoglobulin-secreting cells

A series of experiments was performed to measure the number of IgM-, IgG-, IgA- and IgE-SC in normal healthy subjects (Table 1). The results show that the mean number of IgA-SC was higher than the mean number of IgG-SC, which again was higher than the mean number of IgM-SC. This distribution was statistically significant ($P < 0.001$). It is also evident from the table that the number of IgSC differs considerably between individuals. In normal individuals IgE-SC occur in less than 1 per 10^6 MNC.

Dermatophagoides-specific antibody-secreting cells

Using the assay conditions found to be optimal in the murine ELI-spot assay together with the anti-human immunoglobulin reagents found optimal for detection of human IgSC, an assay for detection of *Dermatophagoides* antigen specific AbSC was developed.

Table 2 shows the results obtained from three donors undergoing immunotherapy with *D. pteronyssinus* extract. The table shows between 45 and 76 *D. pteronyssinus*-specific AbSC per 10^6 MNC, with IgA being the dominant isotype. Normal donors did not exhibit any circulating *D. pteronyssinus*-specific AbSC. These results demonstrate that it is possible to measure allergen-specific AbSC to *Dermatophagoides* antigens after immunotherapy.

Discussion

A simple and specific method has been developed for counting allergen-specific human AbSC. These cells are difficult to obtain, especially in numbers suitable for optimizing an assay. Therefore *Der p* I-specific hybridoma cell lines were used to optimize the different assay parameters and also to describe the limitations of the assay. The NHP4 cell line was fully characterized with respect to antibody affinity coefficient and specificity, including epitope specificity, and furthermore was available in unlimited quantities [9]. From the experiments performed with murine hybridoma cell lines it is known that antibodies with affinity coefficients of at least $1.2 \times 10^9 \text{ M}^{-1}$ are detected [9].

Antigen specificity was demonstrated by a dose-dependent inhibition of spot formation by the addition of specific antigen, but not by an irrelevant antigen. However, despite rather high concentrations of antigen the inhibition was not total. This was probably caused by the experimental design in which the sedimented cells were lying in close contact with the allergen-coated plastic surfaces. This was in accordance with the finding that the remaining spots were smaller than the spots from the uninhibited wells.

Inhibition experiments with the protein synthesis inhibitor cycloheximide showed that spot formation was dependent on active synthesis and secretion of immunoglobulin.

In addition to the specific ELI-spot assay it was of interest to measure the total number of circulating IgM-, IgG- and IgA-SC. This assay should function as a positive control to the specific assay and also describe any potential change in the number of activated B cells as a response to immunotherapy. This assay was implemented to count circulating IgSC in normal healthy donors (Table 1). The results are comparable with those found by us earlier using a traditional reverse type haemolytic plaque forming cell assay [16].

Table 1 shows that the mean number of IgA-SC (850 per 10^6 MNC) is significantly higher than the mean number of IgG-SC (532 per 10^6 MNC). Although IgG is the major immunoglobulin in normal human serum, IgA is the isotype produced in largest quantities, but the majority is found in secretions [17,18]. The number of AbSC in peripheral blood might then reflect the total B-cell activation, and a theory is that the cells may be on the passage to their effector lymphoid tissue [19]. Therefore, measuring the AbSC rather than the plasma antibodies might give a truer impression of the total B-cell activation.

The data presented in Table 2 demonstrate that it is possible to measure circulating *Dermatophagoides*-specific AbSC after immunotherapy. Control subjects did not exhibit any *Dermatophagoides*-specific activation. The results from the experiments performed with the murine hybridoma cell lines secreting antibodies to *Der p* I demonstrated that the number of *Der p* I-specific AbSC agree with the number of IgSC. Therefore, it is expected that the 'human' ELI-spot assay detects all the human circulating *Dermatophagoides*-specific AbSC among the IgSC.

It is intended to use this assay to follow the allergen-specific B-cell response during immunotherapy. In this way knowledge can be obtained about the individual patients' reaction to the treatment. By correlating this to the effect of the immunotherapy further insight may be obtained into the basic immunological mechanisms

behind the efficacy of the treatment.

Until recently the majority of research in this field has concentrated upon specific immunoglobulin in plasma. Nevertheless, it has not been possible to find a clear correlation between the antibody response and the outcome of the treatment. The assay presented makes it possible to describe the response on the B-cell level, and in this way to obtain a different picture of the *D. pteronyssinus*-specific B-cell activation. Plasma immunoglobulin does not correlate to the overall production of immunoglobulin, especially not immunoglobulin specific for antigens presented on various mucosal membranes. As inhalant allergens naturally use this route of immunization this could be the reason for the lack of correlation between plasma isotypes and clinical efficacy of immunotherapy. Therefore, measurement of circulating AbSC instead of circulating antibodies might be more relevant. In this way it might be possible to obtain knowledge about specific antibodies that could otherwise only be obtained from the different mucosal tissues.

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