



Research paper

Detection of recombinant *Alt a1* in a two-site, IgM based, sandwich ELISA opens up possibilities of developing alternative assays for the allergen

Makonnen Abebe^{a,*}, Veena Kumar^a, Soundaram Rajan^a, Ajay Thaker^b,
Serdal Sevinc^a, Hari M. Vijay^{a,*}

^a Healthy Environment and Consumer Safety Branch, Health Canada, Tunney's Pasture, Ottawa, ON, Canada K1A 0L2

^b Health Products and Food Branch, Health Canada, Tunney's Pasture, Ottawa, ON, Canada K1A 0L2

Received 11 July 2005; received in revised form 2 March 2006; accepted 2 March 2006

Abstract

Alternaria alternata is well known to induce IgE-mediated asthma in humans. *Alt a1*, a 29 kD glycoprotein doublet composed of 14.5 and 16 kD subunits, is the major allergen of this mould. Detection of *Alt a1* relies on a two-site sandwich ELISA using the same IgG subclass immunoglobulin as primary and secondary antibody. In this study, we have compared two IgM monoclonal antibodies against recombinant and native *Alt a1* in detecting the allergen in a two-site sandwich ELISA. Although both IgM clones detected the native and the recombinant allergen by SDS-PAGE immunoblotting and by the antibody-capture ELISA, only the IgM against recombinant *Alt a1* was able to detect the corresponding, and not the native allergen, in a two-site sandwich ELISA. The IgM against native *Alt a1* was unable to detect either allergen by this method. A combination of the two IgM clones and with a commercially available IgG failed to detect both allergens. However, atopic human IgE detected both forms of the allergen with the two IgM clones as primary antibody. This is the first time to demonstrate detection of *Alt a1* in a two-site, IgM based, sandwich ELISA opening up possibilities for exploring novel detection methods, based on this approach.

© 2006 Elsevier B.V. All rights reserved.

Keywords: *Alternaria alternata*; Recombinant and native *Alt a1*; IgM against native *Alt a1*; IgM against recombinant *Alt a1*; Two-site sandwich ELISA; Antibody-capture ELISA

1. Introduction

The allergenic characteristic of *Alternaria alternata* in inducing IgE-mediated asthma in humans is well

documented (Aas, 1970). Although *A. alternata* is known to possess many allergens (Kroutil and Bush, 1985; Paris et al., 1990; Vijay et al., 1990; Breitenbach et al., 1995) the cDNA library of 9 of them, approved by the Allergen Nomenclature Committee, namely: *Alt a1* (28 kD), *Alt a3*, *Alt a4* (57 kD), *Alt a5* (45 kD), *Alt a6* (11 kD), *Alt a7* (22 kD), *Alt a8* (29 kD), *Alt a10* (53 kD), and *Alt a12* (11 kD) have been completely sequenced (Vijay and Kurupp, 2003).

Alt a1 has been purified in its native state and has also been cloned and expressed in *Pichia pastoris* (Curran et

* Corresponding authors. Inhalation Toxicology and Aerobiology Section, Environmental Health Directorate, F.G. Banting Building, Rm 264, Health Canada, Tunney's Pasture, Ottawa, ON, Canada K1A 0L2, Postal Locator 2202D2. Tel.: +1 613 957 0962/954 9647; fax: +1 613 941 6959.

E-mail addresses: Makonnen_Abebe@hc-sc-gc.ca (M. Abebe), Hari_Vijay@hc-sc-gc.ca (H.M. Vijay).

al., 1993; De Vouge et al., 1996, 1998) in our laboratory as well as by other investigators (Achatz et al., 1995). Extensive studies have shown that this allergen has a molecular weight of about 29 kD (Curran et al., 1993; De Vouge et al., 1996, 1998; Achatz et al., 1995; Deards and Montague, 1991; Yunginger et al., 1980; Aden et al., 1999). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced condition has revealed the allergen to be a doublet composed of 14.5 and 16 kD subunits. *Alt a1* is considered to be a major allergen of *A. alternata* due to the fact that the majority of atopic sera, up to 92%, have been shown to possess IgE antibodies against it (De Vouge et al., 1998). *Alt a1* is currently used as a reference standard for the development of ELISA kits to detect specific IgE antibodies in the sera of atopic patients and to quantify *A. alternata* fungal allergens in house dust samples (Asturias et al., 2003; Portnoy et al., 1998; Vailes et al., 2001; Duffort et al., 2002).

Unlike most other allergens which depend on a primary and a secondary labelled antibody targeted against two different epitopes, the fact that *Alt a1* is a doublet has made it relatively easier to use the same specific antibody i.e. primary as well as the secondary antibody in a two-site sandwich ELISA indicating that the allergen possesses the same target epitope on the 2 subunits.

Currently in our laboratory, mouse monoclonal (MAb) and rabbit polyclonal (PAb) antibodies against recombinant (r-*Alt a1*) and native *Alt a1* (n-*Alt a1*) have been developed and evaluated. During the screening process of potential clones, two antibodies belonging to the IgM class — one raised against the recombinant (IgM-R) and the other against the native (IgM-N) allergen were both observed to possess a very strong reaction to both forms of the allergen. This prompted further investigations using various immunological techniques to gather more information in their interaction with the allergen in the recombinant as well as the native state to determine their application in the development of diagnostic kits for the detection of the allergen in house dust samples.

2. Material and methods

2.1. Production and isotyping of monoclonal antibodies (MAbs)

The production of MAbs against r-*Alt a1* was carried out using the ClonaCell monoclonal antibody production kit purchased from StemCell Technologies (Vancouver, Canada). *P. pastoris* expressed r-*Alt a1* (De

Vouge et al., 1996) purified by FPLC was used to immunize Balb/c mice. Clones were initially screened by the antibody-capture ELISA, described below. Positive clones were isotyped by the InstantCHEK kit (StemCell Technologies). The production of monoclonal antibodies against n-*Alt a1* was carried out with column chromatography-purified (Curran et al., 1993) *Alt a1* from crude extract of *A. alternata* spores. Clones were isolated by limiting dilution and screened by the antibody-capture ELISA, described below, in 96 well plates coated with n-*Alt a1* at 100 µg/well. Positive clones were isotyped using the Sigma-Aldrich isotyping kit. In both experiments, Balb/c mice were used for immunization and Sp2/O/Ag14 myeloma cells were used for fusion with spleen cells from immunized mice.

2.2. In vitro production and purification of MAbs

The two IgM clones that were strongly reactive to recombinant and native *Alt a1*, in the antibody-capture ELISA, were initially amplified in 10% FBS supplemented D-MEM in 75 cm² cell culture flasks (NUNC, USA) followed by a gradual adaptation process to animal component-free medium (BD Biosciences, USA). The adapted MAbs were further amplified in pure animal component-free medium and transferred into a CL-1000 bioreactor flask (Integra Biosciences, UK) at a density of 1.5×10^7 /ml and incubated at 37 °C in 5% CO₂ incubator. The MAbs were harvested after 21 days following the 21/21 BD Biosciences protocol that was acquired from the manufacturer's website. Gradual addition of solid ammonium sulfate at 0.36 g per ml over a 30 min period was used to precipitate the MAbs on an ice bath with constant stirring. The precipitated MAbs were centrifuged for 30 min at 7000 rpm in a Sorvall RC 5C Plus ultracentrifuge (Sorvall, USA) at 4 °C using the SS-34 rotor. The supernatant was discarded and the pellet was re-suspended in PBS pH 7.4. One wash with PBS pH 7.4 was made using an Amicon filter with a 100 kD molecular weight cut-off membrane (Millipore, USA). The amount of total protein was determined using the BCA protein assay kit (PIERCE, USA) with the aid of a Beckman–Coulter DU 640 spectrophotometer (Beckman–Coulter, USA). Both clones were further isotyped using the InstantCHEK kit.

2.3. Biotinylation of MAbs

Five milligrams of each MAb was used for biotinylation. The EZ-Link Sulfo-NHS-LC biotinylation kit (PIERCE) was used according to the

manufacturer's instructions. The biotinylated antibodies were concentrated with the aid of a 100 kD molecular weight cut-off Amicon filter (Millipore) and re-suspended in 50% glycerol/distilled de-ionized H₂O and aliquots of 100 µl were stored at –20 °C until needed.

To determine the sensitivity of the biotinylated antibodies, serial dilutions of 1/10 to 1/10,000 were prepared and tested using the antibody-capture ELISA with the slight modification of directly adding horseradish peroxidase labelled streptavidin (St-HRP) (1/1000) after adding the biotinylated antibodies to the allergen coated wells followed by washing 4× with phosphate buffered saline 0.05% Tween-20, pH 7.4 (PBS-T) and adding 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) substrate, (ABTS), (ZYMED, USA). Four time-frame readings at OD₄₀₅ were made for each dilution at 5, 10, 15, and 20 min intervals. The titre that gave a reading of 2.0 at OD₄₀₅ within 15 to 20 min was determined from a graph constructed with the four time-frame readings of each dilution.

2.4. ELISA

For all ELISA assays, the Immulon 2HB 96 well plates (Thermo Lifesciences, USA) were used. The source of n-*Alt a1* was a crude extract of the ATCC-34-016 isolate of *A. alternata* with a 20% total protein content. One and five nanograms per well of r-*Alt a1* were used in the antibody-capture and two-site sandwich ELISAs respectively; while 100 µg per well of n-*Alt a1* was used in both assays. These quantities were initially determined to yield reliable readings (data not shown) for the batch of allergens used in this study.

Washing of plates was done 4× with 0.05% Tween-20 in 10 mM phosphate buffered saline (PBS-T) pH 7.4 at 200 µl per well while the same amount of 1% bovine serum albumen in 10 mM PBS (1% BSA/PBS) pH 7.4 was used for blocking at room temperature for 2 h. Coating of plates either with antibody or allergen was done with 10 mM PBS pH 7.4 at 100 µl/well followed by incubation at 4 °C overnight unless otherwise stated. In the two-site sandwich assays, the allergen was suspended and serially diluted in 1% BSA/PBS pH 7.4. In two-site sandwich ELISA assays, ABTS and a 1/1000 dilution of St-HRP were added at 100 µl/well. In the antibody-capture ELISA 3, 3', 5, 5'-tetramethylbenzidine (TMB) (KPL, USA) was used at 100 µl/well. An automated ELISA reader (MRX Revelation, DYNEX Technologies, USA) was utilized for absorbance readings.

2.4.1. Antibody-capture ELISA

Twelve pairs of wells coated with n-*Alt a1* and another 12 pairs coated with r-*Alt a1* were treated as follows: After washing and blocking, 1 µg protein per well of IgM-N was added to half of the wells coated with n-*Alt a1* while the same amount of IgM-R was added to the remaining half. The wells coated with r-*Alt a1* were similarly treated and the plate was incubated for 1 h at RT. Horseradish peroxidase (HRP) labelled secondary goat anti-mouse antibody (heavy and light chain) (KPL, USA) was added, after washing, followed by incubation for another hour at RT. The absorbance was read at OD₄₅₀ after adding TMB substrate and stopping the reaction with 1 M phosphoric acid.

2.4.2. Two-site sandwich ELISA

Half of 12 pairs of wells were coated with 1 µg per well of IgM-N and the remaining half with IgM-R. Another 12 pairs of control wells were coated with a commercially available IgG MAbs against *Alt a1* (Indoor Technologies, USA) according to the manufacturer's instructions. Recombinant *Alt a1* was added in half of the wells coated with IgM-N and n-*Alt a1* was added to the remaining half. The wells coated with IgM-R and the control wells were treated in the same manner and the plate was incubated for 1 h at RT. The wells coated with the test IgMs were further divided into 3 groups of 2 pairs of wells. To each group, biotinylated IgM-N, IgM-R and IgG antibodies were added, while biotinylated IgG was added to the control wells. St-HRP was added after washing followed by 30 min incubation at RT and the absorbance was read at OD₄₀₅ after adding ABTS.

2.4.3. A sandwich ELISA to detect *Alt a1* using a combination of IgM-N/R and atopic IgE

Six pairs of wells were coated with IgM-R while another 6 pairs were coated with IgM-N at 1 µg protein per well. Recombinant *Alt a1* was added in the wells coated with IgM-R, while n-*Alt a1* was added in the wells coated with IgM-N. This was followed by washing and adding 100 µl of 1% BSA/PBS per well. One hundred microlitres of a pool of atopic sera, initially diluted 1/100, was added to each of the first pair of the 6 pairs of well followed by serial dilution leaving the last pair for base-line control. The same was repeated for the remaining 6 pairs coated with the IgM-N. Biotinylated mouse anti-human IgE (Upstate, USA) was added at a 1/500 dilution followed by another hour of incubation at RT. Addition of St-HRP, ABTS, and absorbance readings was essentially the same as the two-site sandwich ELISA. The OD readings were graphically analysed using the Prism graphic software (GraphPad, USA).

2.4.4. Construction of an IgM-R based *Alt a1* assay standard curve

Twelve pairs of wells were coated with 1 µg protein per well IgM-R. After washing and blocking, 100 µl of 1% BSA/PBS was added to each well. The procedure for the two-site sandwich ELISAs was followed after adding 250 ng r-*Alt a1* suspended in 100 µl 1% BSA/PBS to each of the first pair of wells and serially diluting and leaving the last 2 pairs of wells as base-line control. The experiment was repeated 3× and the data collected from the ELISA readings was used for the automatic construction of a sigmoid standard curve by the application software (Revelation 4.02, DYNEX Technologies, USA).

2.4.5. IgM capture ELISA

The assay procedure for the IgM capture ELISA was essentially the same as that of the two-site sandwich ELISA except for the fact that the plate was initially coated with rabbit polyclonal IgG against mouse IgM (Sigma-Aldrich, USA) at 1 µg/well and incubating for 2 h at 37 °C and blocking followed by the addition of the test IgM antibodies and incubating overnight.

2.5. SDS-PAGE and immunoblotting

The reactivity of the two MAbs was tested with n-*Alt a1* as well as r-*Alt a1* using SDS-PAGE immunoblot under reduced using 1% dithiothreitol (DTT), and non-reduced conditions. Twenty-five micrograms of total protein was loaded on a 12% polyacrylamide gel in discontinuous buffer (Laemmli, 1970) and proteins separated on SDS-PAGE gel were transferred onto PVDF membrane (BioRad Laboratories) using semi-dry Transblot system (BioRad Laboratories). The membrane was blocked with 2% skim milk powder in Tris-buffered saline for 2 h at RT followed by incubation with the test MAbs (1 mg) at 4 °C overnight. The membrane was incubated with goat anti-mouse conjugated with HRP for 2 h after 3× washes with TBST followed by incubating with opti-4CN substrate (BioRad Laboratories) after another 3× washes. Normal mouse serum was used as control.

3. Results

3.1. Isotyping and in vitro production of MAbs

The two selected clones were confirmed to belong to the IgM class of antibodies. From the in vitro propagation of the clones, 16 ml of both antibodies was harvested from the corresponding CL-1000 biore-

actor flasks. The amount of antibody produced in vitro was 2.5 mg/ml for IgM-R while that for IgM-N was 2.23 mg/ml. Therefore, a total of 40 and 35.68 mg of MAbs were collected for those raised against the recombinant and native allergen respectively. This is in agreement with most observations made on in vitro production of MAbs using the CL-1000 bioreactor flask and the animal component-free medium. Repeated isotyping of the two MAbs after amplification in the CL-1000 bioreactor flask confirmed the initial observations.

3.2. Biotinylation of MAbs

The activity of the biotinylated antibodies at a concentration of 1/500 gave an OD reading of over 2.00 at 405 nm. Therefore, this dilution of the biotinylated IgMs was used for both antibodies in the sandwich ELISA test.

3.3. ELISA tests and immunoblotting

Both antibodies were able to detect *Alt a1* in the recombinant as well as the native state in the antibody-capture ELISA and immunoblotting under reduced and non-reduced conditions (Fig. 1). Although the IgM-R

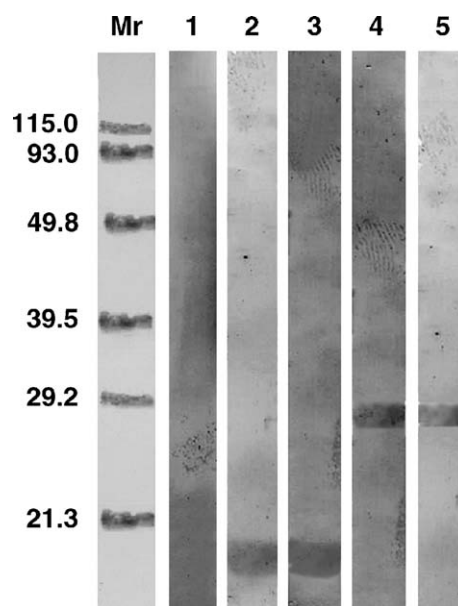


Fig. 1. Immunoblots of recombinant and native *Alt a1* using IgM-R and IgM-N under reduced and non-reduced conditions. 1—Normal mouse serum. 2—IgM-R under reduced condition. 3—IgM-N under reduced condition. 4—IgM-R under non-reduced conditions. 5—IgM-N under non-reduced conditions. Mr—Molecular weight marker.

Table 1

Showing the detection of recombinant and native *Alt a1* by IgM-R, IgM-N, IgG and atopic IgE using various immunological assays

Immunological test	IgM to r- <i>Alt a1</i>		IgM to n- <i>Alt a1</i>	
	r- <i>Alt a1</i>	n- <i>Alt a1</i>	r- <i>Alt a1</i>	n- <i>Alt a1</i>
Sandwich ELISA	+	–	–	–
Antibody-capture ELISA	+	+	+	+
Immunoblotting	+	+	+	+
Sandwich ELISA (with biotinylated anti-IgE)	+	+	+	+
IgM capture ELISA (with 2° biotinylated IgM-R)	+	–	–	–
IgM capture ELISA (with 2° biotinylated IgM-N)	–	–	–	–
IgM capture ELISA (with 2° biotinylated IgG)	–	–	–	–

was able to detect the r-*Alt a1* in the two-site sandwich ELISA, it was not able to detect n-*Alt a1* in the same test. On the other hand, even though the IgM-N was able to detect r-*Alt a1* as well as n-*Alt a1*, by immunoblotting and by the antibody-capture ELISA, it was unable to detect either form of the allergen in the two-site sandwich ELISA. Attempts to detect the allergen using a combination of the two IgM antibody clones as primary and as secondary antibody, as well as a secondary IgG, in the two-site sandwich ELISAs turned out to be negative (Table 1).

3.4. Detection of *Alt a1* using IgM-N/-R and atopic IgE in a sandwich ELISA

Detection of *Alt a1*, in a two-site sandwich ELISA using IgM-R and IgM-N as primary antibodies, and atopic IgE, revealed that the assay was able to detect the allergen. A simultaneous decline in the OD values corresponding to a decrease in the IgE titre in the test sera was observed (Fig. 2).

3.5. Construction of a standard curve using IgM-R as primary and secondary antibody

Repeated construction of a protein assay standard curve in order to determine the stability and reproducibility of an IgM-R based assay revealed that a reproducible and stable assay comparable to that constructed using commercial IgG can be made using IgM-R as primary as well as secondary antibody to quantify r-*Alt a1* (Fig. 3).

4. Discussion

The observation that IgM-R and IgM-N are able to detect the allergen in the antibody-capture ELISA would suggest that both antibodies are probably detecting the same epitope in one of the subunits where it is easily accessible. Similarly, it can be argued that the second

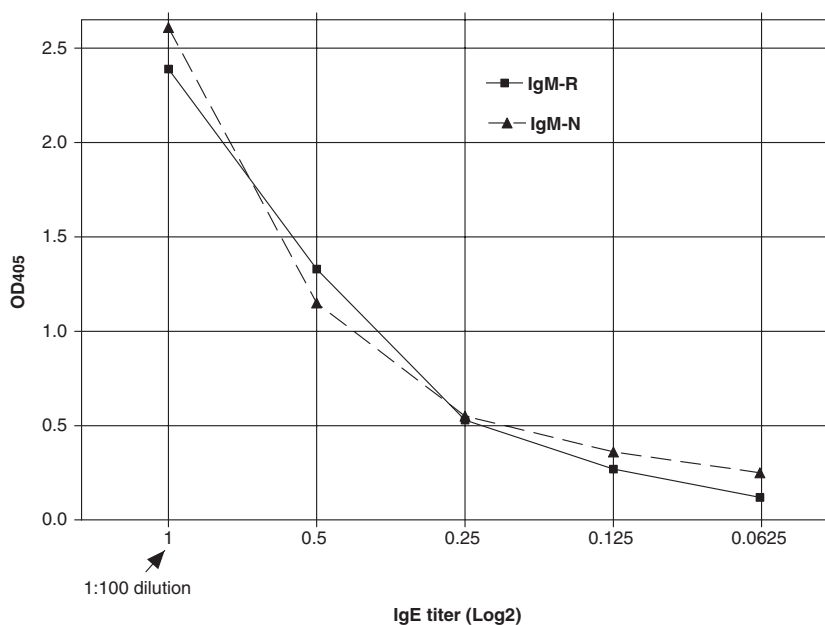


Fig. 2. Graph showing the results of a sandwich ELISA using atopic IgE with IgM-R and IgM-N as primary antibody to detect r-*Alt a1* and n-*Alt a1* respectively.

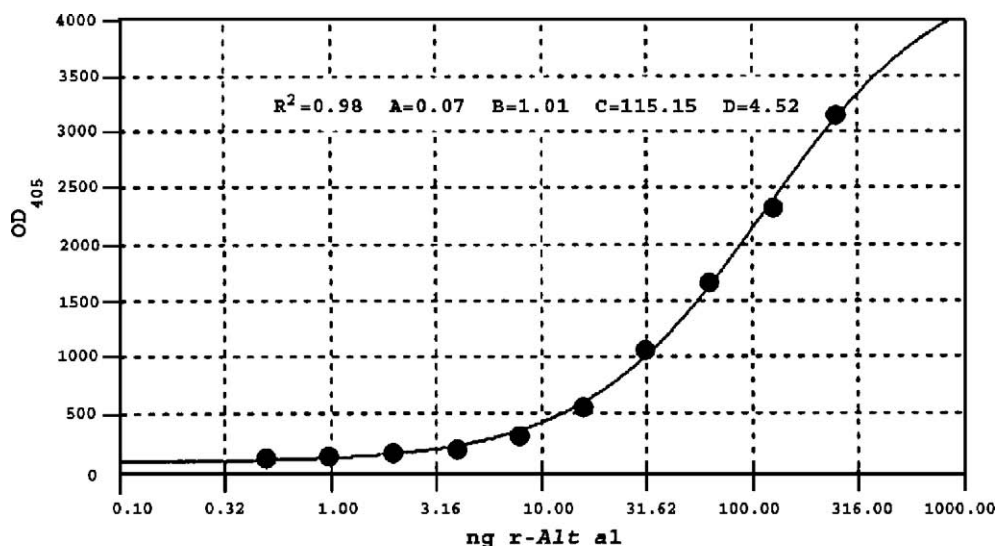


Fig. 3. A typical (sigmoid) protein assay standard curve constructed using IgM-R as primary and secondary antibody with r-Alt a1.

epitope is highly likely to be identical to the first epitope and located on the second site, is inaccessible to both IgMs when the allergen is in the native state. However, the fact that the IgM-N is unable to detect the allergen in the recombinant state using the two-site sandwich ELISA would indicate that this particular antibody is probably recognizing a different epitope than the one recognized by IgM-R and that the epitope on the other subunit of the allergen becomes either inaccessible in the recombinant state or probably does not exist at all.

The IgM capture ELISA is widely used for the diagnosis of various arboviral, parasitic and other infectious diseases. In this assay the presence of virus or parasite specific IgM in patients' sera or cerebrospinal fluid is detected. This technique has given fairly consistent results by utilizing either recombinant target proteins or inactivated whole organisms and biotinylated, monoclonal or polyclonal, secondary IgG in a sandwich ELISA. Using the same IgM capture principle and biotinylated IgG, our attempt to detect the allergen in either state in the present study was unsuccessful. One possible explanation for failure of the IgM capture ELISA to detect the allergen, using biotinylated IgG, could be that the epitope that this antibody is directed against becomes inaccessible when the allergen is bound to the IgM possibly due to steric hindrance. Since the IgM capture ELISA is a slight variation of the two-site sandwich ELISA, steric hindrance could as well be playing the same role in the regular two-site sandwich ELISA. In light of the fact that IgG is by far smaller than IgM, it is more of a surprise to observe the detection of r-Alt a1 using IgM-R as secondary antibody.

In the majority of studies where the IgM capture ELISA has been successfully used, either a relatively high molecular weight recombinant coat protein, approximately 82 kD (WNV) (Wang et al., 2001), or inactivated whole virus (Ebola) (Ksiazek et al., 1999), have been used. It can, therefore, be argued that the target proteins under these situations are large enough and that any steric hindrance from the IgM would be insignificant. Also, the secondary IgG in some cases are polyclonal and could be detecting epitope/s that are not affected by steric hindrance from the IgM. Even though Alt a1 is a relatively small molecule, it is a doublet and therefore it could be speculated that epitopes to the biotinylated IgG should easily be accessible at least on one of the subunits. The fact that a study was able to demonstrate that the relatively small monomeric recombinant P35 antigen (35 kD) of *T. gondii* can easily be detected using the IgM capture ELISA and a monoclonal IgG (Suzuki et al., 2000) would suggest that the conformation of the Alt a1 in either state makes the IgG epitope, inaccessible to the biotinylated IgG. It is interesting to note, however, that the IgE epitope in both forms of the allergen is easily accessible to the corresponding antibody in pooled atopic human sera. This should not be surprising in light of the fact that IgE in atopic sera is polyclonal (Peng et al., 1997) indicating that there could be different IgE epitopes in the r-Alt a1 and n-Alt a1 where the interaction between the epitopes and the IgE paratope is unaffected by steric hindrance from the primary IgM. By the same token, there could be epitopes that are recognized by other monoclonal or

polyclonal IgG and monoclonal IgM in both forms of the allergen.

The discrepancy observed in the detection of the allergen in the native state using any combination of the two IgM antibodies or the commercially available IgG is likely to arise from the difference in the tertiary protein folding in both forms of the allergen. Also, being glycoprotein, other post-translation modifications on the glycosylation of r-*Alt a1* could have made it dissimilar to n-*Alt a1* thus contributing to the differences observed in the over all interaction of the allergen in its different forms with the antibody used in this study.

5. Conclusion

Although IgM may not be considered as a suitable candidate for a two-site sandwich ELISA due to its size (~900 kD), this study has demonstrated that an IgM based sandwich assay was able to detect r-*Alt a1*. The reproducibility of the standard curve constructed, using the IgM-R, conclusively confirms that a two-site sandwich ELISA based on this antibody could be developed to detect and quantify *Alt a1*. Detection of the allergen using atopic sera indicates the possibility that polyclonal or monoclonal IgG, directed against the IgE epitope, could be utilized to target it as a secondary site in an IgM based sandwich assay. Currently, work is in progress to develop assays that quantify native as well as recombinant *Alt a1* using IgM as primary and secondary antibody and a combination of IgM and monoclonal and/or polyclonal IgG antibodies.

Acknowledgements

The authors would like to thank Drs Ivan Curran and Santokh Gills for their editorial assistance and for providing constructive suggestions during the writing-up of the manuscript.

References

- Aas, K., 1970. Bronchial provocation tests in asthma. *Arch. Dis. Child* 45, 221.
- Achatz, G., Oberkofler, H., Leuchenaue, E., Simon, B., Unger, A., Breitenbach, M., 1995. Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Mol. Immunol.* 32, 213.
- Aden, E., Weber, B., Bosser, J., Teppke, M., Frank, E., Wahl, R., Fiebig, H., Cromwell, O., 1999. Standardization of *Alternaria alternata*: extraction and quantification of alt a1 by using an mAb-based 2-site binding assay. *J. Allergy Clin. Immunol.* 104, 128.
- Asturias, J.A., Arilla, M.C., Ibarolla, I., Eraso, E., Gonzales-Rioja, R., Martine, A., 2003. A sensitive two-site enzyme linked immuno-
- sorbent assay for measurement of the major *Alternaria alternata* Alt a1. *Ann. Allergy, Asthma, & Immunol.* 90, 529.
- Breitenbach, M., Achatz, G., Oberkofler, H., Simon, B., Unger, A., Leuchenaue, E., Kandler, D., Ebner, C., Kraft, D., 1995. Molecular characterization of *Cladosporium herbarum* and *Alternaria alternans*. *Int. Arch. Allergy Immunol.* 107, 458.
- Curran, I.H.A., Young, M.N., Burton, M., Vijay, H.M., 1993. Purification and characterization of Alt a-29 from *Alternaria alternata*. *Int. Arch. Allergy Immunol.* 102, 267.
- Deards, M.J., Montague, A.E., 1991. Purification and characterization of a major allergen of *Alternaria alternata*. *Mol. Immunol.* 28, 409.
- De Vouge, M.W., Thaker, A.J., Zhang, L., Muradia, G., Rode, H., Vijay, H.M., 1996. Isolation and expression of a cDNA encoding an *Alternaria alternata* Alt a1 subunit. *Int. Arch. Allergy Immunol.* 111, 385.
- De Vouge, M.W., Thaker, A.J., Zhang, L., Muradia, G., Rode, H., Vijay, H.M., 1998. Molecular cloning of Ig-E binding fragments of *Alternaria alternata* allergens. *Int. Arch. Allergy Immunol.* 116, 261.
- Duffort, O., Barber, D., Polo, F., 2002. Quantification assay for the major allergen in *Alternaria alternata*, Alt a1. *Allergol. Immunol. Clin.* 17, 162.
- Kroutil, L., Bush, R.K., 1985. Detection of *Alternaria* allergens by Western blotting. *J. Allergy Clin. Immunol.* 175, 174.
- Ksiazek, T.G., West, C.P., Rollin, P.E., Jharing, P.B., Peters, C.J., 1999. ELISA for the detection of antibodies for Ebola viruses. *JID* 179, S192.
- Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680.
- Paris, S., Fitting, C., Latge, J.P., Guinépain, M.T., David, P., 1990. Comparison of conidial and mycelial allergens of *Alternaria alternata*. *Int. Arch. Allergy Appl. Immunol.* 92, 1.
- Peng, Z., Arthur, G., Rector, E.S., Kierek-Jaszczuk, D., Simons, F.E., Becker, A.B., 1997. Heterogeneity of polyclonal IgE characterized by differential charge, affinity to protein A, and antigenicity. *J. Allergy Clin. Immunol.* 100, 87.
- Portnoy, J., Brothers, D., Pacheco, F., Landuyt, J., Barnes, C., 1998. Monoclonal antibody-based assay for Alt a1, a major *Alternaria* allergen. *Ann. Allergy, Asthma, & Immunol.* 81, 51.
- Suzuki, Y., Ramirez, R., Press, C., Li, S., Parmley, S., Thulliez, P., Remington, J.S., 2000. Detection of immunoglobulin antibodies to P35 antigen of *Toxoplasma gondii* for serodiagnosis of recently acquired infection in women. *J. Clin. Microbiol.* 38, 3967.
- Vailes, L., Sridhara, S., Cromwell, O., Weber, B., Breitenbach, M., Chapman, M., 2001. Quantitation of the major fungal allergens, Alt a1 and Asp f1, in commercial allergenic products. *J. Allergy Clin. Immunol.* 107, 64.
- Vijay, H.M., Burton, M., Young, M., 1990. The hypoallergen of *Alternaria alternata* carries a dominant epitope of the major allergen. In: Sehon, A.H., Kraft, D., Kunkel, G. (Eds.), *Epitopes of Atopic Allergens. Proceedings of Workshop, XIVth Cong. Eur. Acad. Allergol. Clinical Immunol.*, Berlin Sept 1989. UCB Institute of Allergy, Brussels, p. 14.
- Vijay, H.M., Kurupp, V.P., 2003. Fungal allergens, In: Lokey, R.F., Bukantz, S.C. (Eds.), *Allergens and Allergen Immuno-Therapy*, 3rd ed. Marcel Dekker Inc., New York, p. 223.
- Wang, T., Anderson, F., Magnarelli, L.A., Wong, S.J., Koski, R.A., Fikrig, E., 2001. Immunization of mice against West Nile virus with recombinant Envelop protein. *J. Immunol.* 167, 5273.
- Yunginger, J.W., Jones, R.T., Nesheim, M.E., Geller, M., 1980. Studies on *Alternaria* allergens: III Isolation of a major allergenic fraction (ALT-I). *J. Allergy Clin. Immunol.* 66, 138.