

Isolation and Immunological Characterisation of Allergens from Salivary Glands of the Australian Paralysis Tick *Ixodes holocyclus*

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Abstract. Radioimmunoassay and Western blot analysis showed that salivary gland extract (SGE) prepared from *Ixodes holocyclus* was a more concentrated source of allergens than whole body extract. Isolation and partial immunological characterisation of allergens was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of SGE, electroelution and Western blotting. Immunoblotting using allergic sera showed that partially purified SGE from *I. holocyclus* contained two common allergens of approximately 28 and 35 kilodaltons and several minor allergens. The two most common allergens gave radioimmunoassay ratios significantly greater than the less common allergens. The immunological purity of electroeluted allergens was confirmed by further SDS-PAGE, silver staining and Western blotting.

Introduction

The Australian paralysis tick, *Ixodes holocyclus*, causes several diseases in animals and humans. They include the transmission of rickettsial diseases [1, 2], neuromuscular paralysis [3-6] and hypersensitive responses. In humans, allergic reactions range from mild local pruritis to nausea, hypotension and anaphylaxis [7-10]. People living in, working in or visiting forestry reserves, national parks, or any tick-infested areas are thus a high-risk group.

A radioimmunoassay (RIA) to detect *I. holocyclus*-specific IgE and thus identify hypersensitive individuals is described elsewhere [11]. Skin testing for diagnostic purposes and allergen-specific immunotherapy may also be required by allergic individuals. Currently, immunotherapy to *I. holocyclus* involves the use of crude whole body extract (WBE). Studies on allergies induced by the Hymenoptera (bees, hornets and wasps) have shown (1) that the relevant allergens occur in the venom rather than the body, and (2) that desensitisation with pure venom is superior to WBE [12-17]. It is important, therefore, to determine whether *I. holocyclus* allergens are also concentrated in the salivary glands and saliva rather than the body of the tick. This would confirm previous evidence that

allergens derived from the salivary glands were secreted in saliva, through the tick's hypostome when feeding [18].

Materials and Methods

Preparation of Tick Allergens

WBE (50 µg tick protein/ml) was prepared from unfed *I. holocyclus* females as previously described [11]. Protein concentration was determined by the method of Bradford [19]. For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), frozen tick homogenate containing approximately 330 µg protein was placed directly into 1 ml of hot sample buffer and held at 95 °C for 15 min. Sample buffer contained 4% w/v SDS, 15 mM Tris/HCl in 50% glycerol, pH 8.0, and 4% v/v tracking dye [20]. The resulting suspension was centrifuged at 15,000 g for 15 min at 4 °C and the precipitate discarded.

Salivary gland extract (SGE) was prepared from unfed female *I. holocyclus*. Ticks were attached ventrally to Petri dishes with a small drop of Supa Glue®, covered with sterile normal saline and held at 4 °C on a refrigerated 'cold table' during microdissection. Salivary glands were removed and stored at -20 °C until required. For RIA, glands were thawed and homogenised in an all-glass hand homogeniser in 0.1 M carbonate/bicarbonate buffer pH 9.6 at 4 °C. The resulting gland suspension was centrifuged at 15,000 g for 10 min at 4 °C and diluted with buffer to 50 µg protein/ml. For electrophoresis, 20 frozen glands (16.5 µg protein/gland) were placed into 1 ml of sample buffer at 95 °C and treated as for WBE.

Sera

Blood was obtained by venipuncture and sera prepared from volunteers as previously described [11].

SDS-PAGE

500 µl each of SGE or WBE (330 µg/ml) was loaded onto a 3.3–20% gradient gel [20]. 50 µl of prestained low molecular weight standards (BRL, Gaithersburg, Md.) 14.3–200 kilodaltons (kd), was also loaded. Electrophoresis was performed for a total period of 3 h, at 125 V initially until the sample had entered the stacking gel, and then at 200 V.

1.5-mm preparative non-gradient 12% gels were cast and run using a Mini Slab Cell (Bio-Rad). Electrophoresis stock solutions and buffers were prepared according to the procedure of Laemmli [21]. 250 µl of WBE or SGE (330 µg/ml) was loaded onto each gel. Electrophoresis was carried out at 100 V until the sample had entered the stacking gel and then for 200 V, 45 min in total. Wider 12% gels were also cast using the SE600 series vertical slab gel unit (Hoefer, San Francisco, Calif.). Stock solutions and buffers were as for the Mini Protean II Dual Slab Cell, except that 3 liters of running buffer was required and 1 ml tick extract (330 µg/ml) was loaded. SDS-PAGE was performed at 7 W per gel for 45 min.

Western Transfer

I. holocyclus proteins separated by SDS-PAGE were transferred to 0.45 µm nitrocellulose membranes (Schleicher and Schuell, Dassel, FRG). Transfer was performed in 25 mM ethanolamine-40 mM glycine buffer containing 20% methanol, pH 9.5, for 18 h at 4°C [22].

Detection of IgE-Reactive Allergens

Nitrocellulose membranes were blocked with 20 ml of a 1% solution of six pooled control sera in PBS, pH 7.5, for 1 h at 37°C. Membranes were cut into 5-mm strips and immersed in sera from allergic or non-allergic volunteers, diluted 1:10, 2 ml per strip. Strips were then reacted successively with goat anti-human IgE (Bio-Rad), 2 µg/ml, followed by rabbit anti-goat IgG (Dakopatts, Denmark), 20 µg/ml, and goat peroxidase-anti-peroxidase (PAP; Dakopatts), 20 µg/ml. The substrate was chloro-1-naphthol [23]. All steps after blocking were performed for 2 h at 23°C. Dilutions were made in 1% control sera, with 2 ml reagent solution per strip. Washing was performed, after blocking, between steps with 0.05% Tween 20 in PBS, three times for 5 min per wash. IgE-reactive material was visualised as dark bands after reaction with substrate.

Estimates of molecular weights were made by standard methods [24, 25] modified by taking the square root transformation of distance travelled to enhance linearity of the regression [J. Kerr, unpubl. method].

Protein Elution

I. holocyclus proteins were visualised in SDS-PAGE gels after electrophoresis by staining according to the procedure outlined in the Model 422 Electroeluter instruction manual (Bio-Rad). The proteins corresponding to the intense bands appearing on Western blots were assumed to be putative allergens. They were excised from the gel and sliced into 5 × 2 mm sections. Allergens were eluted from gel slices, 6 mA per protein band for 2–3 h in 25 mM Tris-192 mM glycine and 0.1% (w/v) SDS, pH 8.6. Eluted proteins (400–600 µl) were concentrated in Centricon 10 Microconcentrators (Amicon, Danvers, Mass.) by centrifugation for 30–45 min at

Table I. Comparison by RIA of IgE-binding capacities of WBE (50 µg protein/ml) and SGE (50 µg protein/ml) from the Australian paralysis tick *I. holocyclus*

Serum ¹	WBE		SGE	
	cpm ²	R ³	cpm ²	R ³
C.W.	3,866 ± 42	3,118	5,303 ± 50	5,003
R.S.	3,459 ± 53	2,790	6,025 ± 114	5,684
L.B.	1,124 ± 47	906	2,779 ± 47	2,622
A.S.	1,563 ± 43	1,260	2,341 ± 73	2,208
S.R.	984 ± 28	794	1,557 ± 43	1,469
A.Y.	2,802 ± 86	2,260	3,159 ± 42	2,980
E.W.	2,891 ± 140	2,331	2,941 ± 93	2,774
Con ⁴	124 ± 12	100	106 ± 2	100

¹ Sera were collected from 7 allergic individuals.

² Each value is the mean of 3 readings for each serum ± SE.

³ RIA ratio = $\frac{\text{mean value} \times 100}{\text{negative control}}$ [27].

⁴ Control sera prepared from a pool of 6 non-allergic individuals.

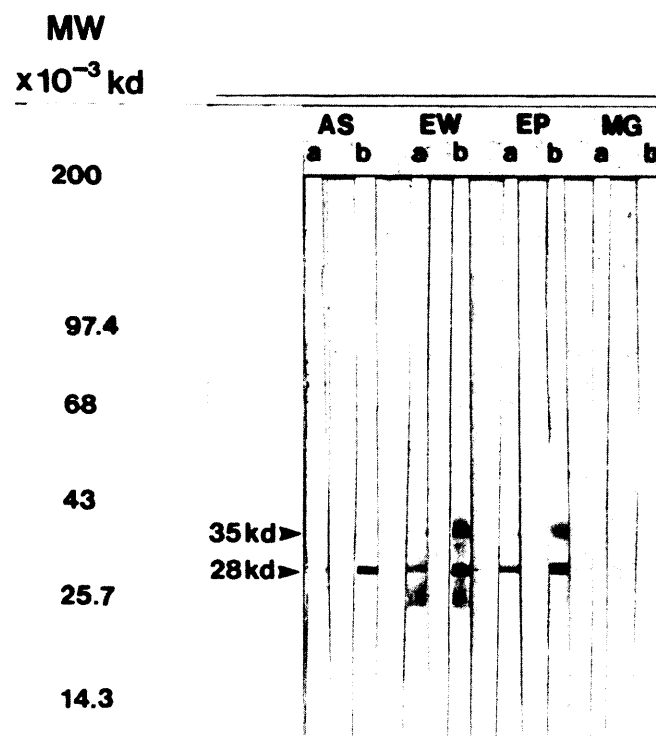


Fig. 1. Comparison by Western blot analysis of IgE-binding capacities of whole body extract and salivary gland extract from the Australian paralysis tick *I. holocyclus*. WBE (a) and SGE (b) allergens after SDS-PAGE on a 3.3%–20% gradient gel and transfer onto nitrocellulose. Visualisation of allergen bands was by PAP. AS, EW, EP: sera from allergic volunteers; MG: negative control serum.

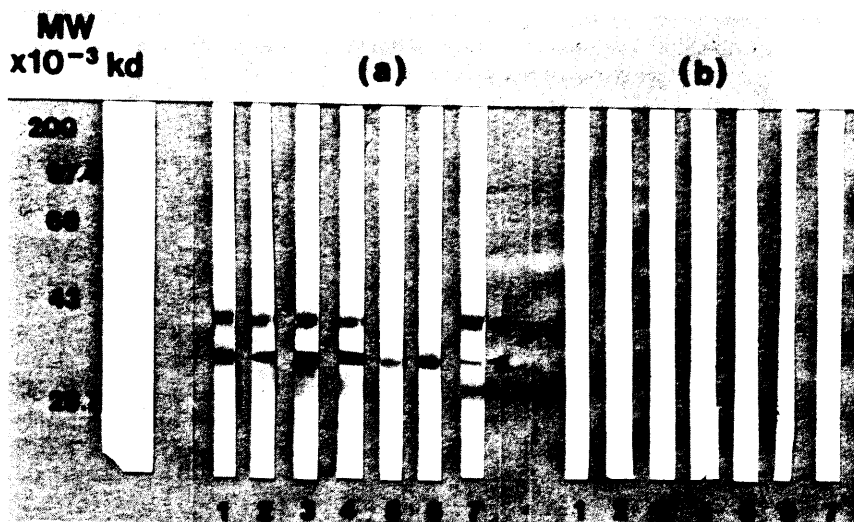


Fig. 2. Immunoblots of salivary gland extract from the Australian paralysis tick *I. holocyclus*. Seven known allergic patients (a) and 7 non-allergic control patients (b).

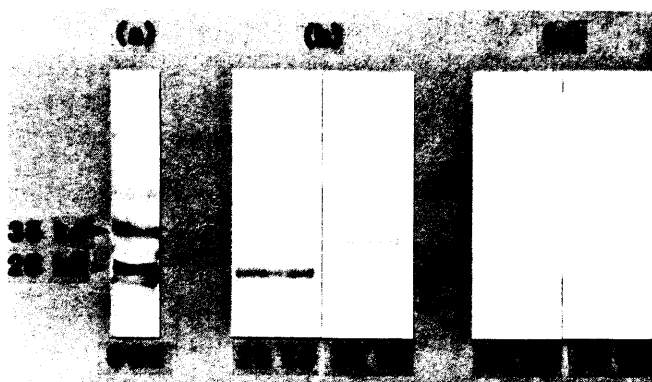


Fig. 3. Immunoblots of SGE (a) and electroeluted 28- and 35-kD allergens (b, c) tested against allergic serum (a, b) and control serum (c).

3,200 g to a final volume of 100–150 μ l. Silver staining (Tsang's modification of Merril et al. [26]) and Western transfer were performed on electroeluted allergens diluted 1:4 to ensure that they corresponded to the proteins excised from the gel (see fig. 3).

Radioimmunoassay

RIA was performed as described previously [11]; except that either SGE or WBE (50 μ g/ml) was coated onto the microtitre plate. RIAs on allergen fractions electroeluted after SDS-PAGE were modified as follows because of the small quantities of protein available. 10 μ l of each eluted allergen (50 μ g/ml) in elution buffer was coated onto the microtitre plates; 25 μ l of serum diluted 1:2 in 1% BSA-PBS was applied. Finally, 10 μ l of radio-iodinated anti-human IgE was added. Incubation times and temperatures and washing were as described elsewhere [11]. Blocking was with 100 μ l 1% BSA-PBS. Bound IgE was measured in counts per minute (cpm) and expressed as a ratio:

$$\frac{\text{mean serum reading} \times 100}{\text{negative control}}$$

Results were classified as negative (<250% of control value), borderline (250–400% of control value) or positive (>400% of control value) according to the method of Homburger and Jacob [27]. RIA ratios were analysed using a paired t test.

Results

Comparison of WBE and SGE

RIA ratios of sera from 7 allergic patients were higher ($p < 0.01$) when SGE was compared as the source of allergens against WBE (table I).

Immunoblots of *I. holocyclus* WBE or SGE allergens showed that IgE-reactive bands were more intense if SGE was used (fig. 1). Therefore, SGE was used as the source of allergens for all subsequent experiments.

Detection of Allergens by Immunoblotting

Sera were tested from 14 patients who had been previously separated by RIA and skin test into equal numbers of allergic and non-allergic individuals. There were two proteins of approximately 28 and 35 kD which reacted with most allergic sera and several including 45-, 50- and 55-kD proteins which reacted with few sera (fig. 2).

Isolation and Partial Purification of Allergens

The silver stain and immunoblot of the 28- and 35-kD electroeluted allergens (fig. 3, 4) show two single bands. They corresponded with the bands which were believed to be putative allergens seen in SGE on gels and Western blots. Therefore, isolation



Fig. 4. Silver stain of SGE and electroeluted 28- and 35-kd allergens. Molecular weight standards $\times 10^{-3}$ kd.

Table II. Comparison by RIA of IgE-binding capacities of five salivary gland allergens isolated from salivary gland extract of the Australian paralysis tick, *I. holocyclus*

Serum ²	Salivary gland allergen RIA ratio ¹				
	28 kd ³	35 kd	45 kd	50 kd	55 kd
A	1,006	553	232	348	219
B	1,444	398	222	286	251
C	865	344	229	241	81
D	4,200	3,349	111	1,081	742
E	802	856	422	359	225
F	1,267	1,381	597	608	450
G	1,675	1,831	754	810	600
H	865	344	229	241	81
I	1,095	1,216	402	430	362

$$^1 \text{ RIA ratio} = \frac{\text{mean RIA value} \times 100}{\text{negative control}} \quad [27].$$

Mean RIA value $\pm$ SE data omitted to simplify the table.

² Sera were collected from 9 allergic volunteers.

³ Approximate molecular weights of purified allergens in kd: 28 and 35 kd (most common allergens); 45, 50 and 55 kd (less common allergens).

and partial purification of the allergens of interest had been effected.

RIA Ratios

The two most common allergens (28 and 35 kd) gave RIA ratios significantly greater ($p < 0.001$) than the less common allergens (45, 50 and 55 kd) (table II).

Discussion

I. holocyclus is a biting tick which secretes vasoactive substances, toxins and allergens into humans and animals when feeding [28, 29]. In common with biting and stinging insects, it may be conjectured that allergens are likely to be concentrated in salivary glands rather than in the whole body of the tick.

In this paper, the problem was addressed as follows: (1) a preparation of SGE was compared, by RIA, with WBE having an equivalent total protein content to determine whether IgE-binding allergens were concentrated in *I. holocyclus* salivary glands. Homogenisation of these glands would liberate saliva contained in the salivary ducts as well as allergen-releasing granules present in the gland acini [18, 29]. (2) Allergens comprising SGE were partially purified by

SDS-PAGE and immunologically characterised by immunoblotting with sera from a panel of allergic and non-allergic volunteers. (3) Isolation of partially purified allergens was achieved by electroeluting five putative allergens detected in (2) above. (4) Finally, the immunological purity and successfulness of the isolation of the electroeluted *I. holocyclus* putative allergens were assessed by performing SDS-PAGE, silver staining and immunoblotting to ascertain that each allergen formed a single protein band on the stained gel and a single IgE-reactive band on the nitrocellulose membrane. IgE-binding capacities of the electroeluted allergens were assessed by RIA.

RIA and Western blot results suggested that allergens were concentrated in the salivary glands. This is analogous to the hymenopteran venom sacs where most allergenic activity resides [15].

Immunoblots showed that allergic sera reacted more frequently with the 28- and 35-kd allergens than with other allergens. It seemed likely, therefore, that the former allergens contributed significantly to *I. holocyclus*-induced hypersensitivity. This hypothesis was supported by the significantly greater RIA ratios which occurred with electroeluted 28- and 35-kd proteins compared with other allergens.

In summary, two proteinaceous allergens of 28 and

35 kd which appear to contribute significant IgE-binding activities on immunoblots and RIA have been isolated from *I. holocyclus* salivary glands. It is likely that after further purification, they may be of clinical use in skin testing and in the successful immunotherapy of *I. holocyclus*-induced allergy, in a manner analogous to hymenopteran-purified venom.

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