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An immunoblot technique for identification of allergens of the Australian paralysis tick *Ixodes holocyclus*

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The blocking conditions appropriate for Western blot identification of allergens of the Australian paralysis tick, *Ixodes holocyclus*, have been investigated. The results suggest that human serum is a potent blocking agent which may have wide application in the immunodetection of antigens.

Key words: Western blot; Blocking agent; Non-specific binding; *Ixodes holocyclus*; Allergen

Introduction

Two major and three minor allergens have been identified in extracts of salivary glands from the Australian paralysis tick *Ixodes holocyclus*, having molecular weights of 28 000 and 35 000 and 45 000, 50 000 and 55 000, respectively (Gauci et al., 1988b). A test for the presence in human serum of antibodies to these allergens would be of value in supplementing the standard skin test used in diagnosing hypersensitivity to this tick. It has been shown that the Western transfer or immunoblot technique of Towbin et al. (1979) is a convenient method for detecting the occurrence of antibodies in the serum of patients suspected of allergy to a number of specific allergens (see for example: Tovey and Baldo, 1984, 1985; Bengtsson et al., 1985; Ford et al., 1985, 1986). This paper reports

the development of an immunoblot method for assisting the diagnosis of allergy to *I. holocyclus* and in determining the frequency of reactivity to individual allergens. Naive human serum has been identified as a potent blocking agent for use in the test to eliminate non-specific reactions.

Materials and methods

Preparation of allergens for electrophoresis

Salivary glands were removed aseptically from unfed female *I. holocyclus* and stored at -20°C until required. 1 ml of electrophoresis sample buffer at 95°C . (4% w/v sodium dodecyl sulphate (SDS), 15 mM Tris-HCl in 50% v/v glycerol, pH 8.0, and 4% v/v tracking dye (Tsang et al., 1983) was added to 20 frozen glands containing $16.6\text{ }\mu\text{g}$ protein/gland (Bradford, 1976). The mixture was incubated at 95°C for 15 min, centrifuged at $15000\times g$ for 15 min, and the precipitate discarded. Salivary glands were used rather than whole body extract because it has been shown that allergens are concentrated in the glands (Gauci et al., 1988b).

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Sera

Blood was obtained by a pathology laboratory from seven allergic and seven negative control subjects by venipuncture and sera prepared by standard methods and adopting standard precautions for operator protection. Subjects were tested by skin prick test and the sera were assessed by RIA (Gauci et al., 1988a) in order to permit their designation as 'positive' or 'negative' prior to use in immunoblotting (data not shown). Negative sera were obtained either from naive subjects or individuals who had been exposed to *I. holocyclus* but were not allergic and were also negative in an IgG EIA test (M. Gauci, B.F. Stone and Y.H. Thong, unpublished data).

Electrophoresis

Preparative 12% SDS-polyacrylamide mini gels were cast using the Mini Slab Cell (Bio-Rad, Richmond, CA). Stock solutions and buffers were essentially those described by Laemmli (1970). 250 μ l of salivary gland extract (SGE) were loaded onto each gel (330 μ g tick protein/ml) and 20 μ l prestained molecular weight markers were also loaded (BRL, Gaithersburg, MA). Electrophoresis was performed at 100 V initially until the sample had entered the stacking gel and then at 200 V for about 45 min.

Western blot transfer

After electrophoresis, SGE proteins were transferred to 0.45 μ m nitrocellulose membranes (Schleicher & Schuell, Dassel, F.R.G.) as described in detail elsewhere (Towbin et al., 1979; Tsang et al., 1983). The transfer buffer used was either 25 mM Tris-193 mM glycine, pH 8.4 or 25 mM ethanolamine-40 mM glycine, pH 9.5. Both buffers contained 20% methanol. Transfer conditions were 60 V and 22 V for Tris-glycine and ethanolamine-glycine respectively, with a run time of 18 h at 4°C.

Silver stain

Silver staining was performed on gels to check the amounts of protein left after Western blot transfer. The standard method of Merrill et al. (1981) as modified by Tsang et al. (1983) was used.

Probing of nitrocellulose membranes to detect specific IgE

After transfer was complete, nitrocellulose membranes were cut into 0.5 mm strips and placed in separate slots in a small incubation tray (Bio-Rad). Unbound sites on the nitrocellulose were blocked with 5 ml of one of five solutions at 4°C: (1) 1% v/v control serum prepared from a pool of six naive individuals; (2) 0.3% v/v Tween 20; (3) 1% w/v bovine serum albumin (BSA); (4) 5% w/v skim milk powder (Carnation) and (5) 1% v/v horse serum (HS). All blocking agents are routinely used in most laboratories except for the control human serum pool. The diluent was PBS, pH 7.4. All subsequent stages were incubated for 2 h at 25°C on a platform rocker. Dilutions were made with the same blocking agent with which strips were blocked initially. Three 5 min washes in 0.05% v/v Tween 20 in PBS were carried out between steps. After blocking, strips were incubated with (a) positive serum, 1/10, (b) negative serum, 1/10, to control for non-specific binding, and (c) diluent alone to ascertain that the detection of allergen bands were human serum-dependent. Strips were then incubated in goat anti-human IgE used at 2 μ g/ml (Bio-Rad). This anti-serum had been obtained from goats immunised with a purified IgE, κ myeloma protein and the Fc portion of an IgE, λ myeloma protein. Purified rabbit anti-goat IgG, followed by goat peroxidase-anti-peroxidase (PAP) (both obtained from Dako, Denmark, and used at 20 μ g/ml) were then added. Finally, the substrate chloro-1-naphthol was added and allowed to react for 30 min (Hawkes et al., 1982). The strips were rinsed with water and examined for banding.

Determination of molecular weights of SGE allergens

Molecular weights were determined by standard methods (Shapiro et al., 1967; Neville, 1971) modified by taking the square root transformation of the distance travelled to enhance linearity of the regression (J. Kerr, unpublished method).

Results

The choice of blocking agent influenced the banding pattern on immunoblots (Fig. 1). Use of

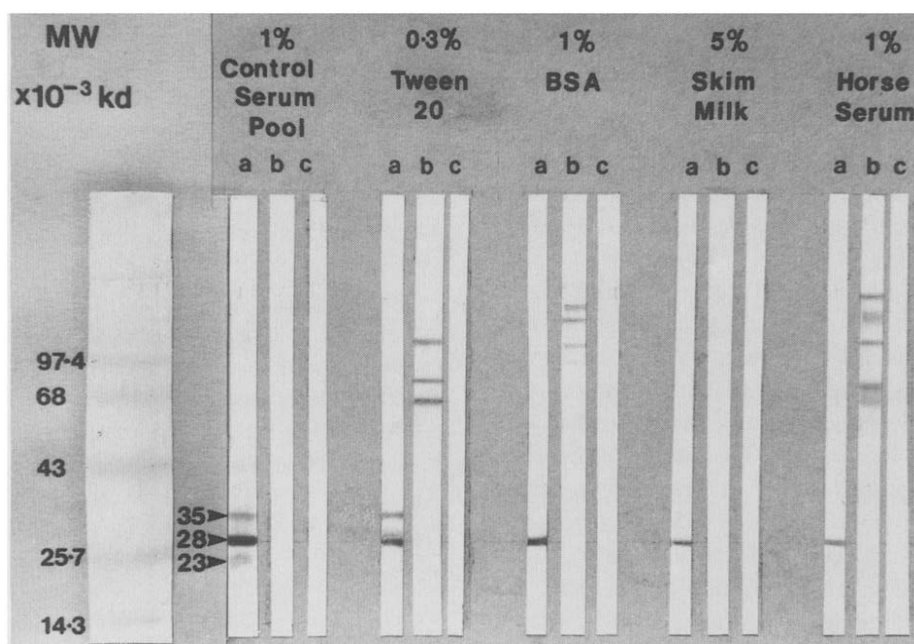


Fig. 1. Effect of five blocking agents on immunoblots of salivary gland allergens from unfed *I. holocyclus* ticks reacted with (a) serum from allergic individuals, (b) pooled control sera and (c) no serum. Note non-specific binding occurs when Tween 20, BSA or HS are used. MW $\times 10^{-3}$ is indicated.

1% v/v naive serum resulted in an intense band of MW 28 000 and two less intense bands of MW 23 000 and 35 000 on the 'a' strips which were allowed to react with 'positive serum', and no bands on 'b' strips which were allowed to react with 'negative' serum. The use of 0.3% v/v Tween 20 resulted in an intense band of Mw 28 000 and a less intense band at MW 35 000 on the 'a' strip. BSA, skim milk powder and HS solutions gave a single intense band of MW 28 000 on strip 'a'. Tween 20, BSA and HS gave 3–5 higher MW bands on strip 'b', ranging from 60 000 to 120 000. None of the blocking agents resulted in the appearance of bands on strip 'c' which was developed in the absence of serum.

Discussion

The choice of blocking agent clearly affected the appearance of the immunoblot. No bands appeared on 'c' strips incubated in the absence of human serum, indicating that binding was human serum-dependent. Non-specific binding occurred if Tween 20, BSA or HS were used since 3–5

bands, ranging from MW 60 000 to 120 000, quite distinct from the low MW bands appearing on 'a' strips were apparent on 'b' strips. High background staining with HS was also noted by Tovey et al. (1986). While skim milk stopped non-specific binding, its incorporation into the system resulted in the loss of at least two bands. Similar findings were reported by Spinola and Cannon (1985) and Tovey et al. (1986).

The occurrence of non-specific binding may be related to the PAP technique. This explanation was suggested by Tovey et al. (1986) who reported the appearance of spurious bands in the presence of control serum. They suggested that components other than the specific antigens on the nitrocellulose sometimes reacted with serum proteins. In our hands, however, the PAP system did not give rise to non-specific binding if blocking was performed with pooled control human sera.

However, there is an anomaly in the results. Non-specific binding occurred on 'b' strips but not on 'a' strips if Tween 20, BSA or HS were used. The same non-specific binding would be expected to occur on both strips if the phenomenon were solely related to the blocking agent.

Non-specific binding sites on the nitrocellulose which were incompletely blocked by Tween, BSA or HS on 'b' strips may have been blocked on 'a' strips by unknown agents in 'positive' human serum. In particular, the high levels of circulating specific IgG present in 'positive' sera (M. Gauci, unpublished data) may have contributed to this improved blocking.

The number and intensity of the bands were also affected by the blocking agent. Although the precise cause remains unclear, various explanations have been offered. These include the loss of antigen from the nitrocellulose, the disruption of antigen-antibody complexes by reagents, and the binding of reagents directly to antigens or antibodies (Spinola and Cannon, 1985; Wedge and Svenneby, 1986; Tovey and Baldo, 1987). It is therefore critical in any immunoblotting work to test several different blocking agents and choose the most suitable one for the particular antigen-antibody system in question. Clearly, control human serum is an ideal blocking agent for our purposes and is without interference from non-specific binding. The MW 28 000 and 35 000 proteins have been shown to be important allergens to tick salivary glands and were very obvious while the rarer, minor MW 45 000, 50 000 and 55 000 allergen bands (Gauci et al., 1988b) did not appear to be reactive with the sera tested.

Preliminary investigations on the effect of variations in the pH of the transfer buffer (Szewczyk and Kozloff, 1985) were also examined. Comparison of ethanolamine-glycine, pH 9.5 and the more conventional buffer Tris-glycine, pH 8.4 showed that more protein remained on the gel after Western blot transfer with the latter buffer system. However, development of bands on the nitrocellulose showed no differences in the number and intensities of the bands according to the buffer used (M. Gauci, unpublished data). More work may be needed to fully investigate a range of transfer buffers for the selection of the most appropriate buffer for the detection of *I. holocyclus* allergens. In the meantime ethanolamine-glycine appears to be a suitable buffer for this purpose.

To conclude, an immunoblot for the identification of antigens important in the allergic reactions induced by *I. holocyclus* was developed. The data indicate that human serum is a potent blocking

agent for use in immunoblot techniques. Thus human serum, which is not used routinely as a blocking agent, may have many other potential applications in the immunodetection of antigens.

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