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Enhanced immunodetection of blotted house dust mite protein allergens on nitrocellulose following blocking with Tween 20

The effect of blocking nitrocellulose membranes with the nonionic detergent Tween 20 on the detection, by protein blotting, of IgE-binding to house dust mite *Dermatophagoides pteronyssinus* allergens has been investigated. Tween blocking led to enhanced immunodetection of allergens despite removal of proteins from the membrane when compared to protein blocking agents which did not displace transferred components. The enhancement varied with the different mite components and, for one in particular, antigen *Der p* II, an increase of more than 100-fold in IgE antibody binding occurred despite a concurrent loss of more than 90 % of *Der p* II from the membrane. Both the enhancement of binding and loss of components from the membrane were dependent upon the time course of blocking and the concentration of Tween used.

1 Introduction

The nonionic detergent Tween 20 (polyoxyethylenesorbitan monolaurate) is commonly used to block nitrocellulose (NC) following protein blotting and prior to immunodetection of dotted or transferred proteins [1-3]. As a blocking agent, Tween is inexpensive, convenient to use, requires only a short time for blocking and frequently produces low backgrounds [4]. Some disadvantages with its use have, however, been reported, including displacement of transferred proteins from the membrane [5] and the appearance of nonspecific binding in some antigen-antibody systems [6-8]. In a study designed to compare the effect of different blocking agents on the immunodetection of proteins immobilized on NC, we found that the highest specific uptakes of antiserum occurred with the nonionic detergents Tween and Nonidet P-40 (NP-40) and not with proteins such as bovine serum albumin (BSA), sera, milk powder and gelatin [9]. Enhanced immunodetection of electroblotted proteins on NC blocked with Tween 20 has now been demonstrated in a study designed to identify IgE-binding proteins from the common house dust mite. These findings draw attention to the possibility that nonionic surface active agents such as Tween may promote similar effects with other blotted proteins when used to block NC membranes prior to the application of antibody or other probes used for the detection of transferred proteins.

2 Materials and methods

2.1 House dust mite extract

Clean dried mite (*Dermatophagoides pteronyssinus*) bodies (1g) (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) were homogenized on ice in 20 mL of PBS for 15 min with a Teflon homogenizer in a glass tube. This preparation was then sonicated intermittently for 5 min, stirred at 0 °C for 1 h, centrifuged for 30 min at 18 000 g, concentrated

by ultrafiltration to 14 mg/mL of protein, and stored at -20 °C. All preparatory steps were carried out in a cold room or with ice cooling. Radiolabelled (¹²⁵I) mite extract was prepared using the Bolton Hunter reagent [10].

2.2 Human sera

Sera from mite-allergic subjects were obtained as described previously [11]. One hundred sera from mite-allergic subjects were used to prepare a pooled serum. Sera were also obtained from human cord blood and from pollen-allergic patients who were not allergic to house dust mite. All sera were tested for mite-specific IgE in a radioallergosorbent test (RAST) using Pharmacia Phadebas RAST *D. pteronyssinus* allergen discs and ¹²⁵I-anti-human-IgE (Kallestad Laboratories, Austin, TX). A mouse monoclonal antibody to the important mite allergen DpX (*Der p* II) was provided by Dr. R. C. Aalberse, Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

2.3 Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransfer to NC

SDS-PAGE [12] was carried out as previously described [13]. 2-Mercaptoethanol was not included in the sample extraction buffer. A total volume of 300 µL containing 1.5 mg of mite proteins was loaded onto each gel and the extract was subjected to electrophoresis. Low *M_r* markers (94, 67, 43, 30, 20, & 14.4 kDa; Pharmacia) were included in each gel. Materials and methods for electrotransfer of separated proteins to NC were as previously described [11, 14] except that total transfer time was extended to 2 h and a variety of different blocking procedures were employed: 3 % BSA (Sigma, 98-99 %, St. Louis, MO), 3 % cold water fish skin gelatin (Sigma) or 5 % skimmed milk powder (Diploma Brand, Unigate Aust., Dandenong, Australia) for 2 h; 0.025 %, 0.1 %, 0.3 %, 1.0 %, or 2.5 % v/v Tween 20 for 1 h; 0.1 % Tween 20 for 1 h at 4 °C, room temperature (RT; 24 °C) or 40 °C; 0.1 % Tween 20 for 15 min, 1 h, 17 h or 24 h. All solutions were prepared in PBS and were at room temperature unless otherwise stated. NC transfers containing the immobilised *M_r* markers were stained with 0.5 % Amido Black (Sigma) in water, methanol, acetic acid 65:25:10 v/v. The experiments with different blocking times and temperatures were performed with different quantities of radiolabelled, but the same quantity of unlabelled, mite

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Abbreviations: BSA, bovine serum albumin; IgE, immunoglobulin E; *M_r*, molecular weight; NC, nitrocellulose; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RAST, radioallergosorbent test; RT, room temperature; SDS, sodium dodecyl sulphate

extract. All experiments were performed at least twice with consistent results.

2.4 Probing of NC membrane strips and autoradiography

After blocking using one of the above procedures, NC strips (5 mm in width) containing separated, unlabelled mite proteins, were incubated overnight with pooled sera or individual sera from mite-allergic subjects (diluted 1:10 with 0.005 % v/v Tween/0.01 % w/v sodium azide/0.5 % BSA w/v in PBS), washed extensively (30 min) with 0.05 % Tween 20 in PBS and incubated overnight with ^{125}I -anti-IgE (80 000 cpm/strip in the 0.005 % Tween-BSA buffer). After washing with 0.05 % Tween in PBS, strips were air-dried and loaded into a Kodak X-omatic cassette (Eastman Kodak Co., Rochester, NY) together with photographic film (Fuji Rx-safety, Tokyo, Japan). The cassette was stored at -70°C for periods of 24 h to 7 days. Similarly, after being blocked, the NC strips containing separated ^{125}I -labelled mite proteins were air-dried and subjected to autoradiography. Following autoradiography the patterns on the photographic film were scanned with a Quick Scan densitometer (Helena Laboratories, Beaumont, TX). Cord sera and sera from subjects not allergic to house dust mites were used as controls. In addition, sections of NC containing protein band *Der p* II (DpX) were subjected to γ -counting in an attempt to more accurately quantitate both the amount of this allergen left on the NC strip and the quantity of specific IgE bound following the various treatments described.

3 Results

3.1 Effect of different blocking conditions on the retention of mite proteins transferred to NC

A comparison was made of the retention of electroblotted ^{125}I -labelled mite proteins on NC blocked with some commonly used protein blocking agents and the nonionic detergent Tween 20. Mite proteins retained on NC were revealed by Amido Black staining (Fig. 1b) or autoradiography following the transfer of ^{125}I -labelled mite protein extract (Fig. 1c). The position of the most important mite allergen *Der p* II was located by reaction with a mouse monoclonal antibody specific for this protein (Fig. 1d). Use of the protein blocking agents BSA (3 %), fish gelatin (3 %) and skimmed milk powder (5 %) for 2 h at RT (24°C) revealed no obvious differences in the number and amount of labelled proteins remaining on the NC strips whether the strips had been blocked (Fig. 1e-g) or unblocked (Fig. 1c). Fig. 1h-k shows the effect of Tween 20 (0.1 %) at RT on the number and amount of retained protein bands when blocking periods of 15 min, 1 h, 17 h and 24 h were employed. Removal from the NC of the separated mite proteins increased with increasing blocking time; for example, over 90 % of bands 1 and 12 (*Der p* II) were removed by the nonionic surface active agent over a 24 h period.

Fig. 1l-p shows the effect of increasing Tween concentration on the removal from NC of mite proteins. As the concentration of Tween increased over the range 0.025-2.5 %, the

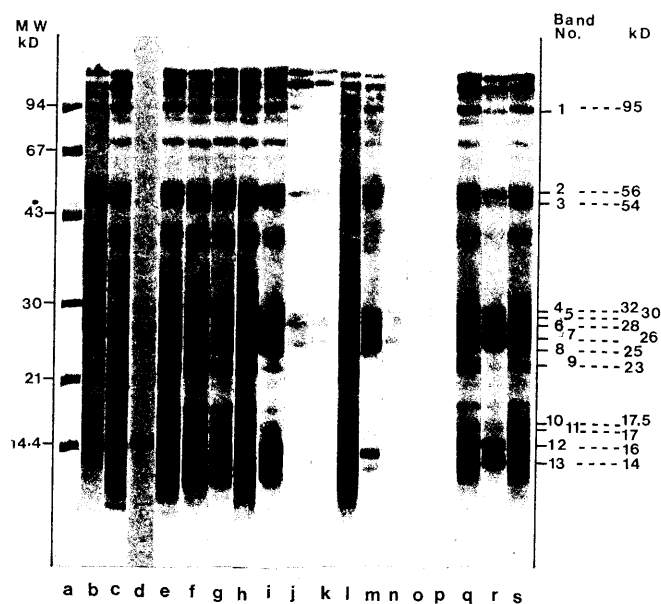


Figure 1. Mite proteins detected by autoradiography after labelling with ^{125}I and following SDS-PAGE, transfer to NC and blocking with Tween or protein blocking agents. (a) Low M_r markers; (b) mite proteins stained for protein with Amido Black; (c) mite proteins detected by autoradiography without prior blocking; (d) strip reacted with anti-*Der p* II mouse monoclonal antibody and detected with ^{125}I -goat anti-mouse IgG heavy and light chains (Cappel, West Chester PA); (e)-(g) mite proteins detected by autoradiography following blocking of strips for 2 h at RT (24°C) with BSA 3 %, gelatin 3 % or skimmed milk powder 5 %, respectively; (h)-(k) strips blocked with Tween 20 0.1 % at RT for 15 min, 1 h, 17 h and 24 h, respectively; (l)-(p) strips blocked for 1 h at RT with Tween 20 0.025, 0.1, 0.3, 1 and 2.5 %, respectively; (q)-(s) strips blocked with Tween 20 0.1 % for 1 h at 4°C , RT and 40°C , respectively. Note: strips (b)-(k) originate from one gel, (l)-(p) from another and (q)-(s) from a third gel. The quantity of radiolabelled mite extract varied between gels but the quantity of unlabelled mite extract was consistent.

amounts of mite proteins removed during the 1 h blocking step increased correspondingly. Some proteins were more easily removed than others. For example, the employment of 0.1 % Tween for 1 h led to the removal of approximately 70 % of component 12 (*Der p* II) but only 27 % of component 2 (56 kDa) (data not shown). The effect of a solution of Tween 0.01 % at three different temperatures, 4°, 20° and 40 °C, on the removal of mite proteins from NC is shown in Fig. 1q-s. All three treatments led to some loss of protein with the greatest loss occurring at 24 °C. This is illustrated by an examination of components 9 and 12 on the NC strips. By subjecting the excised bands to radioactive counting, component 9 showed losses of 92 %, 72 % and 14 % at 24°, 40° and 4 °C, respectively, while component 12 demonstrated losses of 53 % at 24 °C, and 24 % at 4° and 40 °C. Loss of protein from the NC was less marked, compared with control, as the M_r of the proteins increased. No significant losses occurred with components of $M_r > 94$ kDa.

3.2 Detection of IgE-binding proteins on NC following blocking with Tween

Fig. 2 shows the identification by autoradiography of the IgE-binding components on NC following the transfer of SDS-PAGE-separated mite proteins and probing with ^{125} I-anti-human IgE. Fig. 2a, b and c show, respectively, Amido Black stained M_r markers, mite proteins on NC and the allergen *Der p* II located with a mouse monoclonal anti-*Der p* II. Strips d-r were probed with a sample of serum pooled from 100 different house dust mite-allergic subjects. Strips s-v were probed with individual samples of sera from subjects allergic to the mite. Thirteen IgE-binding components were clearly resolved and are numbered on Fig. 2 together with the corresponding M_r . Controls consisting of cord serum and sera from subjects allergic to ryegrass pollen (but not allergic to house dust mites) showed no binding to any of the mite proteins when used with ^{125} I-anti-human IgE as second antibody. Likewise, ^{125} I-anti-

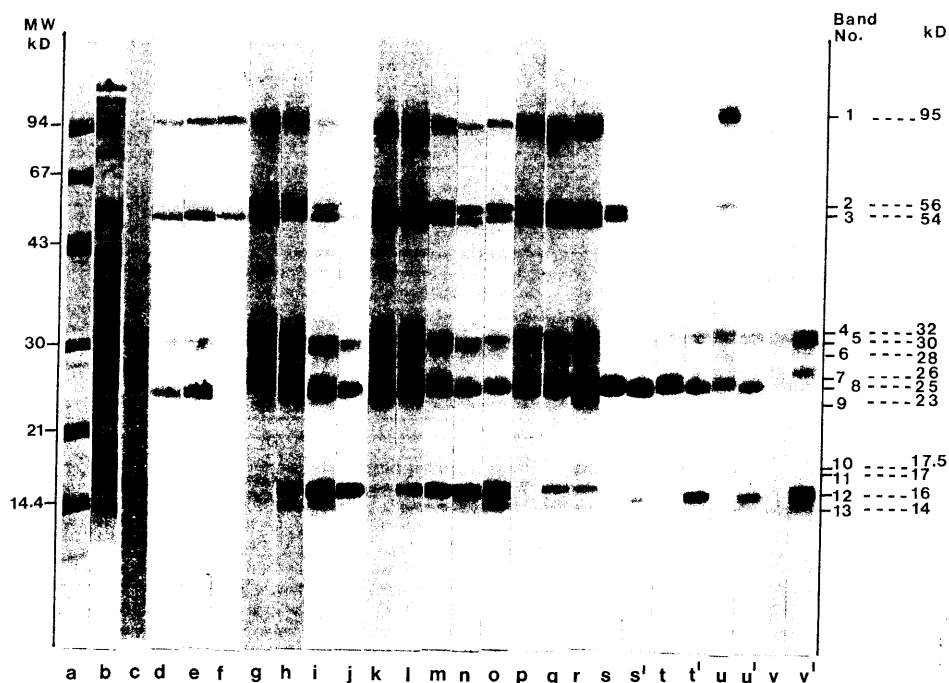


Figure 2. IgE-binding mite proteins detected after transfer to NC and blocking with Tween 20 under various conditions. (a) Low M_r markers; (b) mite proteins stained with Amido Black; (c) mite proteins reacted with mouse anti-*Der p* II and detected with ^{125} I-goat anti-mouse IgG (see legend Fig. 1); (d)-(f) strips blocked for 2 h at RT with BSA 3 %, gelatin 3 % or skimmed milk powder 5 %, respectively, before incubation with serum pooled from mite-allergic subjects followed by ^{125} I-anti-human IgE; (g)-(j) blocked with Tween 20 0.1 % at RT for 15 min, 1 h, 17 h and 24 h, respectively, before incubation with serum pooled from mite-allergic subjects followed by ^{125} I-anti-human IgE; (k)-(o) strips blocked for 1 h at RT (24 °C) with Tween 20 0.025, 0.1, 0.3, 1 and 2.5 %, respectively, before incubation with serum pooled from mite-allergic subjects followed by ^{125} I-anti-human IgE; (p)-(r) blocked with Tween 20 0.1 % for 1 h at 4 °C, RT and 40 °C, respectively, before incubation with serum pooled from mite-allergic subjects followed by ^{125} I-anti-human IgE; (s)-(v) strips blocked with Tween 20 0.1 % for 1 h and then incubated with one of 4 different sera from house dust mite-allergic subjects followed by ^{125} I-anti-human IgE; (s')-(v') as for (s)-(v) but strips blocked with Tween 20 0.1 % for 17 h. Note: strips (b)-(j) originate from one gel, (k)-(r) from another, and (s)-(v) from a third gel.

human IgE alone did not react with any of the separated mite components (data not shown). Strips d-f (Fig. 2) were blocked with BSA, fish gelatin (both 3 %) and skimmed milk proteins (5 %), respectively. Six IgE-binding bands (numbers 1, 2, 3, 4, 5 and 8), all in the M_r range 25-95 kDa, were resolved following blocking with BSA or fish gelatin but only three IgE-binding bands (numbers 1, 2 and 3, M_r range 54-95 kDa) were detected following blocking with the milk proteins.

As in the above-described study with ^{125}I -labelled mite proteins (and as shown in Fig. 1), blocking with 0.1 % Tween 20 was examined over a period from 15 min to 24 h (Fig. 2g-j). After blocking, NC strips were probed with serum containing mite-reactive IgE antibodies and reactive components on the strips were detected with ^{125}I -anti-human IgE. With the clear exception of component 12 (*Der p* II), the intensity of IgE-binding bands detected by autoradiography decreased with increasing incubation time. The intensity of band 12 after a 17 h blocking period increased approximately 5-fold when compared to the result obtained after blocking for only 15 min with Tween solution (Fig. 2g and 2j). Between 17 and 24 h, the intensity of band 12 decreased by only 16 % as measured by counting the excised bands in a gamma-counter. Marked increases (up to 7-fold) in IgE binding to component 12 were also observed in experiments employing increasing concentrations up to 2.5 % Tween 20 for blocking (Fig. 2k-o). Increases, though less marked, were also observed with components 9 (faintly visible on figure) and 13. The other IgE-binding components generally exhibited decreases (up to 75 %) in the intensity of the bands observed on the autoradiographs. The greatest decreases were seen with solutions containing 2.5 % Tween.

The results of blocking with 0.1 % Tween 20 at 4°, 24° and 40 °C are shown in Fig. 2p-r. In general, similar band intensities reflecting IgE-binding were observed but some differences were noted: (i) A complete absence of band 12 when probing was carried out following blocking at 4 °C but not at 24° or 40 °C. This was also observed with component 13

where, however, the effect was less marked. (ii) Binding of IgE to components 10 and 11 at 40 °C but not at the two lower temperatures. (iii) Greater binding of IgE to component 6 at 4° than at 24 °C or 40 °C.

The effect on IgE-binding by mite components following blocking of strips in 0.1 % Tween for 1 h and 17 h are shown in Fig. 2s-v. Probing with sera from 4 different mite-allergic subjects (s, t, u, v) revealed marked increases in the intensity of band 12 after a 17 h blocking period in Tween solution (*cf.* strips s and s', t and t', u and u' and v and v'). The intensities of bands 4, 5 and 7 also increased (see in particular strips v and v') after a 17 h blocking period. By contrast, protein components, of M_r 54 kDa became less distinct or disappeared entirely following the extended blocking period. This finding was similar to the results obtained with the ^{125}I -labelled mite protein transfers (Fig. 1g-j).

3.3 Quantitative examination of Tween enhancement of IgE antibody binding to mite protein *Der p* II (component 12)

An examination of the results summarized in Figs. 1 and 2 clearly reveals Tween-enhanced detection of binding of IgE antibodies to allergenic component 12 (*Der p* II) on NC blots of SDS-PAGE-separated mite proteins even when most of the allergen had been removed from the membrane by the non-ionic detergent. Table 1 lists enhancement ratios for the detection of IgE-binding (as measured by the uptake of ^{125}I -anti-IgE) (see legend of Table) obtained following Tween blocking. When blocking was carried out with 0.1 % Tween for 17 h, IgE antibody binding of *Der p* II as measured by radioactive counting was increased 75 times relative to binding obtained after the use of BSA.

An even greater enhancement (120-fold) was achieved when the NC was blocked with 2.5 % Tween for 1 h. Fig. 3 further illustrates this phenomenon, *viz.* as *Der p* II is progressively removed from the membrane by Tween, the apparent IgE-binding is increased, particularly between 15 min and 17 h.

Table 1. Enhancement of the detection^{a)} of allergen *Der p* II (component 12) on NC following blocking with Tween 20

Blocking procedure ^{b)}	Radioactive counts from component 12 ^{c)} labelled with		Ratio of counts of ^{125}I -anti-IgE: ^{125}I ($\times 100$)	Relative enhancement ratio ^{d)}
	^{125}I	IgE/ ^{125}I -anti-IgE		
BSA 3 %, 2 h	20,795	206	1.0	
Fish gelatin 3 %, 2 h	19,746	282	1.4	
Milk proteins 5 %, 2 h	15,978	24	0.2	
Tween 0.1 %, 15 min	22,693	361	1.6	1
1 h	13,161	846	6.4	4
17 h	2,462	1,833	74.5	46.5
24 h	2,230	1,535	68.8	43
Tween 0.025 %, 1 h	11,726	334	2.8	1
0.1 %, 1 h	3,355	691	20.6	7.4
0.3 %, 1 h	1,368	1,138	83.2	29.7
1.0 %, 1 h	629	1,574	250.2	89.4
2.5 %, 1 h	653	2,189	335.2	119.7

a) *Der p* II (component 12) detected by incubation of NC transfers with sera from mite-allergic subjects followed by incubation with ^{125}I -anti-human IgE and counting excised bands

b) All blocking procedures carried out at RT. Experiments on the effect of blocking time (15 min to 24 h) with 0.1 % Tween used different amounts of radiolabelled mite extract but the same amount of unlabelled mite extract as experiments utilising different Tween concentrations (0.025 % to 2.5 % Tween) for 1 h.

c) Radioactive counts/20 min, minus background, of excised band of component 12 labelled with ^{125}I or reacted with IgE/ ^{125}I -anti-IgE

d) Enhancement ratios for detection of IgE-binding derived by allotting a value of 1 to the ratios of counts (^{125}I -anti-IgE: ^{125}I) obtained with the Tween 0.1 % for 15 min and the Tween 0.025 % for 1 h treatments

Table 2. Enhancement of the detection^{a)} of allergenic components 1, 2, 3 and 5^{b)} on NC following blocking with Tween 20

Blocking procedure ^{c)}	Ratio of bound labels ¹²⁵ I-anti-IgE: ¹²⁵ I ^{d)} for components			
	1	2	3	5
BSA 3 %, 2 h	4.8	1.8	8.6	0.9
Fish gelatin 3 %, 2 h	4.8	0.9	9.5	1.5
Tween 0.1 %, 15 min	8	8.8	13.6	5.4
1 h	7.5	8	14.1	7.8
17 h	16.6	18	105	41.2
24 h	20	13.3	160	53.3

a) Components detected on nitrocellulose with sera from mite-allergic subjects and ¹²⁵I-anti-human IgE and by counting excised bands.

b) Components identified by numbered bands in Figs. 1 and 2

c) All procedures carried out at RT

d) Determined from peak heights of densitometric scans of labelled components on autoradiographs. Figures in the columns are the calculated ratios multiplied by 10

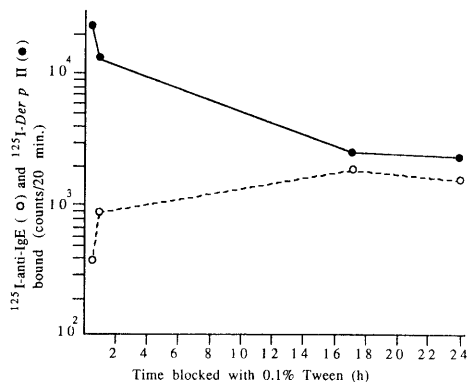


Figure 3. ¹²⁵I-labelled and IgE/¹²⁵I-anti-IgE-labelled *Der p II* detected on NC transfers after blocking the membrane for increasing times in a solution of Tween 20 0.1 %. Although the total amount of bound protein decreased (●) there was a concurrent increase in the amount of IgE-reactive *Der p II* (○) detected.

3.4 Tween-induced enhancement of IgE-binding to other mite proteins

The enhanced detection of IgE-binding to *Der p II* observed following blocking with Tween was not restricted to this allergen but was also observed with other components over a wide *M_r* range. However, unlike the situation with *Der p II*, this enhancement was not obvious from the autoradiographs (Fig. 2d-f and g-v') because the enhancement was not great enough to override the progressive removal of protein that occurred during the Tween incubations. When the ratios of radiolabelled anti-IgE to the radiolabelled component were estimated from densitometric scans of the respective autoradiographs, the enhancement of IgE-binding that occurred with increasing Tween incubation times was evident (Table 2).

4 Discussion

In a previous investigation of IgE-binding to NC-adsorbed nondenatured *Cynodon dactylon* grass pollen extract, we

found that the binding of IgE was greater when non-ionic detergents such as Tween and NP-40 were used as the blocking agent rather than when protein blockers were used [9]. We later demonstrated that more components were detected, and were detected more strongly, when Tween rather than a number of protein blocking agents were used on blots from SDS-PAGs of mite extracts [15]. It was suggested that such nonionic detergents may act by reducing steric hindrance and/or by "deaggregating" the nondenatured proteins to allow better antibody access. Results presented here demonstrate that the role of the blocking agent in immunodetection on NC blots can be more extensive than that of a neutral substance simply blocking unoccupied areas of the membrane. While previous reports from this laboratory [9, 15] and the results of others [7, 8, 16] have shown that the blocking agent employed can influence the detection of specific antigens, this is the first time that enhanced detection of membrane-bound proteins resulting from what appears to be a progressive interaction between the blocking agent Tween and the membrane-bound protein has been demonstrated. The degree of enhancement was related to the incubation time, temperature of blocking and the Tween concentration.

The enhanced detection of protein bands was seen with many, if not all, of the components of mite extracts. However, for one component in particular, of *M_r* 16 kDa and referred to by the standard nomenclature as *Der p II* [17], enhanced detection represented a net gain of up to 120-fold relative to the IgE binding seen when protein blocking agents were used. Incubation of blots with Tween sometimes determined whether or not specific IgE to *Der p II* was detected at all in individual sera. In results published elsewhere [18], an optimised blocking procedure of 0.1 % Tween for 17 h was used for the analysis, by protein blotting, of the rank order of importance of 32 different house dust mite allergens. Antibodies to *Der p II* were demonstrated in 88 % of the sera, making it the most commonly recognised allergen in *D. pteronyssinus* extracts. In an earlier study from this laboratory using a blocking time of 1 h in 0.1 % Tween, the *Der p II* component was recognised by 15 (68 %) of the 22 sera tested [13].

In the present study, enhanced detection also resulted with some other components, although the enhancement was variable and depended upon the component, as shown in Fig. 2. However, because of their simultaneous loss from the membrane, this was not always obvious with some bands. In addition, different Tween blocking conditions may be required for the optimum detection of other components. For example, in incubations with 0.1 % Tween, a blocking time of only 15 min showed the greatest antibody binding to the component of *M_r* 23 kDa while a temperature of 40 °C for 1 h was the most satisfactory for components 10 and 11 (*M_r* 17 and 17.5 kDa). It would appear that the mechanism of Tween-enhancement was dependent on temperature. The effect of a reduction of blocking temperature (1 h, 0.1 % Tween) to 4 °C (Fig. 2, p) was similar to short incubation times (15 min, 0.1 % Tween) (Fig. 2, g) or dilute Tween concentrations (1 h, 0.025 % Tween) (Fig. 2, k) at RT. A previous report of "nonspecific binding" induced by Tween blocking [6] detailed binding by some members of a panel of monoclonal antibodies to some, rather than to all, components on blots. Such "nonspecific binding" can also occur with protein blocking agents and hyperimmune serum. Wedege *et al.* [8], for example, observed such binding by two monospecific sera with different protein components on blots depending on whether BSA or Tween

was used as the blocking agent. The enhancement of IgE binding seen here was not due to Tween-induced nonspecific binding of IgE to the *Der p* II component, as 12 % of 96 sera examined [18] had IgE specific for other mite components but not *Der p* II, and sera which were negative on RAST assay for mite extract, but strongly positive for ryegrass (*Lolium perenne*) pollen, did not show binding to *Der p* II. Additional controls of IgE-free human cord sera and the anti-IgE antibody alone also failed to react with *Der p* II.

Hauri et al. [16] showed that the recognition of antigen by two monoclonal antibodies was dependent upon both the nature and concentration of three different protein blocking agents used. Similarly, Spinola and Cannon [7], using 4 antisera (3 polyclonal and 1 monoclonal) and 5 different blocking/washing treatments, showed that antigen detection was highly dependent upon the treatment used. Blocking with Tween led to a marked enhancement of detection of many antigens compared to procedures employing BSA or milk powder although binding by the monoclonal antibody was only observed when BSA blocking and Tris-saline washes were employed. In our study, the observed loss of components from the membrane during incubation with Tween was consistent with previous observations [5]. Similar losses have been observed with other nonionic detergents such as NP-40 [19] and Triton X [20]. Indeed, the losses which can result (90 % in some cases) are greater than those obtained using agents such as acetonitrile, specifically for the preparative elution of protein from membranes [21]. Losses from membranes with Tween have not always been observed. Bird et al. [16] found no diminution in the intensity of protein staining of NC membranes after 18 h incubation in 0.05 % Tween. It has been demonstrated that subsequent displacement of proteins from the membrane is markedly dependent upon the buffer and detergent conditions under which the subunits first encounter the membranes [22]. While investigating codfish allergen M-IgE interactions by protein blotting, we could not detect the allergen (M_r 12 kDa) on the membrane by either specific immunodetection or protein staining following blocking with Tween, but detection was possible prior to Tween blocking by protein staining and with antibody probes following blocking with proteins (Hemmings and Baldo, unpublished observations).

Several possible mechanisms for Tween-induced enhancement of immunodetection can be suggested. Small molecules such as Tween offer less steric hindrance and a more hydrophilic environment for large antibody molecules to approach antigens on the surface of the membrane. Conversely, protein blocking molecules such as BSA or casein are larger than many of the individual antigens on the membrane surface and may physically restrict antibody access to determinants. The greater enhancement of IgE-binding seen with lower M_r components is consistent with this. A second form of steric hindrance similar to the "prozone" effect commonly seen in radioimmunoassays using a solid-phase bound antigen may also play a role. In this case, excess antigen leads to a reduction in antibody binding, presumably because of poor access to determinants and, as the quantity of antigen decreases, there is an increase in the antibody bound followed by a decline with further antigen dilution. In protein blotting from SDS-PAGEs where denatured proteins are involved, a third possible effect of the blocking agent is renaturation of determinants on the membrane. It is recognised that the denaturation of native extracts for SDS-PAGE with heat, SDS and possible additional reduction of disulphide linkages, involves a progressive reduc-

tion of antigenicity [15]. In particular, conformational determinants associated with the secondary and tertiary molecular structure may be lost. Treatments of the gel prior to transfer with urea [23], and nonionic detergents such as NP-40 [24] or Triton X [25] have all been used in attempts to enhance refolding of the protein, often with only limited success [22]. Following transfer, the extent and the nature of binding and refolding of the SDS-protein complex on the membrane has not been elucidated. It seems likely that Tween, (and other nonionic detergents), can displace SDS from membrane-bound proteins and enhance refolding to a more "native" conformation.

These studies suggest that with Tween, and other blocking agents, it may be necessary to experiment with blocking times, temperatures and concentrations to determine their effects on the immunodetection of individual components. When analysis of specific antibodies to a large number of components is sought, then more than one set of blocking conditions, or a single compromise blocking protocol, may be required.

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