


Antigenic cross-reactivity between *Schistosoma mansoni* and peanut: a role for cross-reactive carbohydrate determinants (CCDs) and implications for the hygiene hypothesis

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Introduction

Schistosomes are parasites belonging to the broad class of invertebrates known as flatworms and responsible for the human disease schistosomiasis, a parasitic disease of public health importance that has chronic, debilitating consequences.^{1,2} Eight species in the genus *Schistosoma* distributed in many tropical countries are known to

infect humans,³ though three species, namely *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* are medically the most important.

Early schistosome infections, before patency, induce host immunity of the T helper type 1 (Th1) type involving interferon- γ and tumour necrosis factor- α .⁴ However, both acute *S. mansoni* infection and sensitization by allergens evoke similar immune responses that are Th2-biased

Summary

The antigenic reactivity of constituents of *Schistosoma mansoni* and peanut (*Arachis hypogaea*) was investigated to determine whether identical antigenic epitopes possessed by both organisms provided a possible explanation for the negative correlation between chronic schistosome infection and atopy to allergens. Aqueous extracts of peanuts were probed in Western immunoblots with rabbit IgG antibodies raised against the egg, cercarial and adult worm stages of *S. mansoni*. Several molecules in the peanut extract were antigenically reactive with antibodies from the various rabbit anti-schistosome sera. A pair of cross-reactive peanut molecules at ~30 000–33 000 molecular weight was purified and both proteins were identified by mass spectrometric analysis as the peanut allergen *Ara h 1*. Anti-*S. mansoni* soluble egg antigen antibodies that were eluted off the peanut molecules reacted with two *S. mansoni* egg antigens identified by mass spectrometry as IPSE/ α -1 and κ -5. Alignments of the amino acid sequences of *Ara h 1* and either IPSE/ α -1 or κ -5 revealed a low level of peptide sequence identity. Incubation of nitrocellulose paper carrying electrophoresed peanut molecules, six constituents of other allergic plants and *S. mansoni* egg antigens in a mild solution of sodium metaperiodate before probing with antibodies, inhibited most of the cross-reactivities. The results are consistent with the antigenic cross-reactive epitopes of *S. mansoni* egg antigens, peanut and other allergic plants being cross-reactive carbohydrate determinants (CCDs). These findings are novel and an explanation based on 'blocking antibodies' could provide an insight for the inverse relationship observed between schistosome infection and allergies.

Keywords: antigenic cross-reactivity; cross-reactive carbohydrate determinants; hygiene hypothesis; IgG antibodies; peanut *Ara h 1*; *Schistosoma mansoni*.

Abbreviations: CCDs, cross-reactive carbohydrate determinants; CFA, complete Freund's adjuvant; IL-4, interleukin-4; MS, mass spectrometry; MW, molecular weight; NMS, normal mouse serum; SmCh, *Schistosoma mansoni* cercariae homogenate; SmSEA, *Schistosoma mansoni* soluble egg antigens; SmWh, *Schistosoma mansoni* adult worm homogenate; Th1, T helper type 1

and characterized by the release of cytokines such as interleukin-4 (IL-4), IL-5 and IL-13, eosinophilia, and production of IgE antibodies.^{5,6} Chronic and advanced stages of infection are characterized also by Th2 immunity, but in a form that is 'modified or 'modulated' and that evokes regulatory T-cell activity and production of cytokines such as IL-10 and transforming growth factor- β , which curb excessive inflammatory responses and so enhance survival of the parasite and perhaps that of the host also.^{4,7,8} Incidentally, the characteristics of immunomodulated anti-parasite Th2 immunity are to some extent similar to those that pertain after successful immunotherapy of allergic diseases.^{9–11}

Several studies indicate that schistosome-infected individuals show lower skin-prick test positivity to allergens,^{12–14} including some plant food allergens.¹⁵ An inverse relationship has been reported between *S. mansoni* infection and allergic sensitization in infected individuals.^{7,16,17} The accumulated evidence is consistent with the hygiene hypothesis, which in summary holds that a reduction in the prevalence of microbial and parasitic infections in people in developed or urban areas and/or improved hygiene and increased rates of vaccination, have resulted in immune responsiveness becoming misdirected and reacting against otherwise innocuous molecules in pollens, food, water and venoms, or indeed against the body's own molecules to give rise to autoimmunity.^{12,18–20}

Chronic infection with schistosomes has been surmised to have an immunoregulatory effect on allergic sensitization and some of the regulatory mechanisms by which this occurs have been discussed.^{11,14,21} One explanation could be induction of immunomodulated responses by chronic parasitic infections, during which regulatory B and T cells are activated to produce anti-inflammatory cytokines such as IL-10 and transforming growth factor- β and the synthesis of IgG4 is initiated.^{14,21} An alternative is the blocking antibody hypothesis: IgG, particularly IgG4, may compete with IgE for common epitopes on allergens.^{9,22–26} IgG-blocking antibody has been reported in patients undergoing allergen-specific immunotherapy, for example after allergen-specific immunotherapy for peanut allergy.²⁷

We have recently used rabbit IgG antibodies to demonstrate antigenic cross-reactivity between molecules in *S. mansoni* egg and cercarial extracts and a latex allergen (*Hev b 7*) and we suggested the possibility that anti-*S. mansoni* IgG antibodies could block allergen-specific IgE reactions.²⁸ The study of antigenic cross-reactivity between *S. mansoni* and allergens was therefore continued here with respect to peanut, which is of global medical importance with a relatively high prevalence.^{29–31}

In this study, peanut constituents were probed for antigenic reactivity with IgG antibodies from rabbits that had been immunized with different stages of *S. mansoni*. Two

peanut molecules that were antigenically cross-reactive with the parasite were identified as isoforms of *Ara h 1*. Consistent with this observation, rabbit anti-*S. mansoni* IgG antibodies reactive with *Ara h 1* reacted with two known *S. mansoni* egg antigens. Most of the cross-reactivity was abrogated by prior treatment with sodium metaperiodate, suggesting that it was due to cross-reactive carbohydrate determinants (CCDs).

Materials and methods

Except when otherwise stated, chemicals, reagents and buffers were bought from Sigma-Aldrich (Poole, UK) and were all of analytical grade.

Preparation of *S. mansoni* antigen/soluble extracts

A Puerto-Rican *S. mansoni* isolate was maintained by continuous passages through *Biomphalaria glabrata* and random-bred CD1 strain mice. Maintenance of the parasite life cycle was performed with strict adherence to the regulations set out in the UK Animals (Scientific Procedures) Act, 1986, (project licence numbers PPL 40/3024 and 40/3595). Animals were killed by administration of a lethal dose of pentobarbitone anaesthetic.

The method of preparation of *S. mansoni* soluble egg antigens (SmSEA) extracted from the livers and intestines of infected mice harbouring adult worms was as described in ref. 32. SmSEA was lyophilized in 1-mg aliquots and stored at -80° .

Preparation of allergen extracts

Peanut extract was prepared from fresh peanut seeds bought from a grocery shop. Extraction of protein was performed as described in ref. 28.

Latex extract was prepared from Copydex (Henkel, Winsford, UK), a commercially obtainable product made from natural rubber-tree latex sap, as described in ref. 28.

All other fruits for preparation of aqueous extracts were bought in ripe form from grocery stores: melon (*Cucumis melo* spp.), banana (*Musa* spp.), avocado (*Persea americana*), tomato (*Lycopersicon esculatum*) and kiwi (*Actinidia deliciosa*). Fruit extracts were prepared as described in ref. 28. Frozen extracts were then lyophilized overnight and stored at -20° .

Preparation of extracts from mouse blood, kidney and spleen

Mouse blood was prepared as described in ref. 33. Extracts of mouse kidney and spleen were prepared by homogenization, sonication and centrifugation of the tissues using the same extraction methods as for the fruits above.

Protein estimation in different extracts

The estimation of protein concentration in all extracts was done using the Bio-Rad DC Protein assay (BioRad, Hemel Hempstead, UK) adapted from ref. 34 using bovine serum albumin as the standard.

Preparation of rabbit polyspecific anti-S. mansoni egg, anti-cercariae and anti-whole adult worm antisera and antisera monospecific for S. mansoni egg antigens

Polyspecific anti-S. mansoni antisera were raised against homogenates of S. mansoni eggs (anti-SmSEA), cercariae (anti-SmCh) and adult worm (anti-SmWh) by immunization of rabbits with the respective homogenates as in ref. 35 and 36.

Rabbit antisera against two egg antigens, interleukin-4-inducing principle from schistosome eggs IPSE/ α -1 and κ -5, were prepared as described in ref. 36.

Rabbit anti-mouse serum and anti-complete Freund's adjuvant antisera

Polyspecific rabbit anti-normal mouse serum (anti-NMS) and anti-complete Freund's adjuvant (anti-CFA) sera were prepared as described in ref. 33 by injecting rabbits with repeated weekly doses of a 1-ml emulsion containing CFA with an equal volume of NMS or isotonic saline.

One-dimensional SDS-PAGE and Western immunoblotting

SDS-PAGE was performed as described in ref. 37, adapted from ref. 38, using 12% acrylamide for the resolving gel and 4.5% acrylamide for the stacking gel. Tris-HCl buffer was used in the preparation of the resolving and stacking buffer solutions and adjusted to pHs of 8.8 and 6.8, respectively.

Western immunoblotting was done as described in ref. 39, adapted as in ref. 36 and 28.

Staining and purification of electrophoresed proteins in SDS-PAGE

SDS-PAGE gels containing electrophoresed proteins were rinsed in distilled water at room temperature three times for 10 min each time. The gels were subsequently covered with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA) for 2 hr for protein staining. Gel de-staining was performed for an hour in several changes of distilled water to allow visualization of protein bands.

Purification of proteins was by excision of the bands of interest from the array of molecules that were present on a de-stained gel, elution of protein from the excised gel band by incubation in buffer (0.06 M Tris-HCl + 10% SDS, pH

7.4) overnight at 37°, centrifugation and re-electrophoresing of the eluate in a second PAGE, as described in ref. 40.

Mass spectrometry and glycosylation analyses of protein samples

Tandem mass spectrometry (MS) was performed on a Waters Corporation Q-TOF 2 instrument^{41,42} according to methods described in ref. 28.

Potential glycosylation sites on amino acid sequences of an MS-identified allergen and two parasite antigens were predicted using the GLYCOEP software⁴³ and the ExPASy Bioinformatics Resource Portal (GlycoMod) tool (<http://web.expasy.org/glycomod/>). The amino acid sequence of the protein was pasted into the software and the prediction based on binary profile of patterns was highlighted while leaving other settings as default and set to run, thereby revealing the number(s) of potential N-linked or O-linked glycosylation site(s) on the amino acid sequence of the protein.

Purification of cross-reactive antibodies by acid elution

Specific anti-SmSEA antibodies that cross-reacted with two peanut molecules were purified by acid elution, a method adapted from ref. 44 and modified as in ref. 28.

Treatment of nitrocellulose paper with sodium metaperiodate

The technique was adapted from ref. 45 and 46 and performed as described in ref. 28.

Results

Cross-reactivity between IgG antibodies in polyspecific anti-S. mansoni sera and peanut antigens

The constituents of an aqueous extract of raw peanuts were investigated in Western immunoblots for their cross-reactivity with five polyspecific rabbit antisera raised against homogenates of three different stages of S. mansoni; two anti-SmSEA antisera, two anti-SmCh antisera and an antiserum raised against adult worm antigens (anti-SmWh). Two rabbit antisera, an anti-CFA and an anti-NMS were used as controls in the same immunoblot.

Figure 1(a) shows that all five rabbit antisera raised against S. mansoni antigens contained antibodies that cross-reacted with many different molecules in the peanut extract with molecular weights (MW) ranging from ~7000 to ~200 000 (lanes 1–5). Neither the anti-NMS nor an anti-CFA sera used as controls in lanes 6 and 7 reacted against any molecules in the peanut extract.

As a control, all five polyspecific anti-S. mansoni sera and the two control antisera (anti-NMS and anti-CFA)

Cross-reactivity between *S. mansoni* and peanut

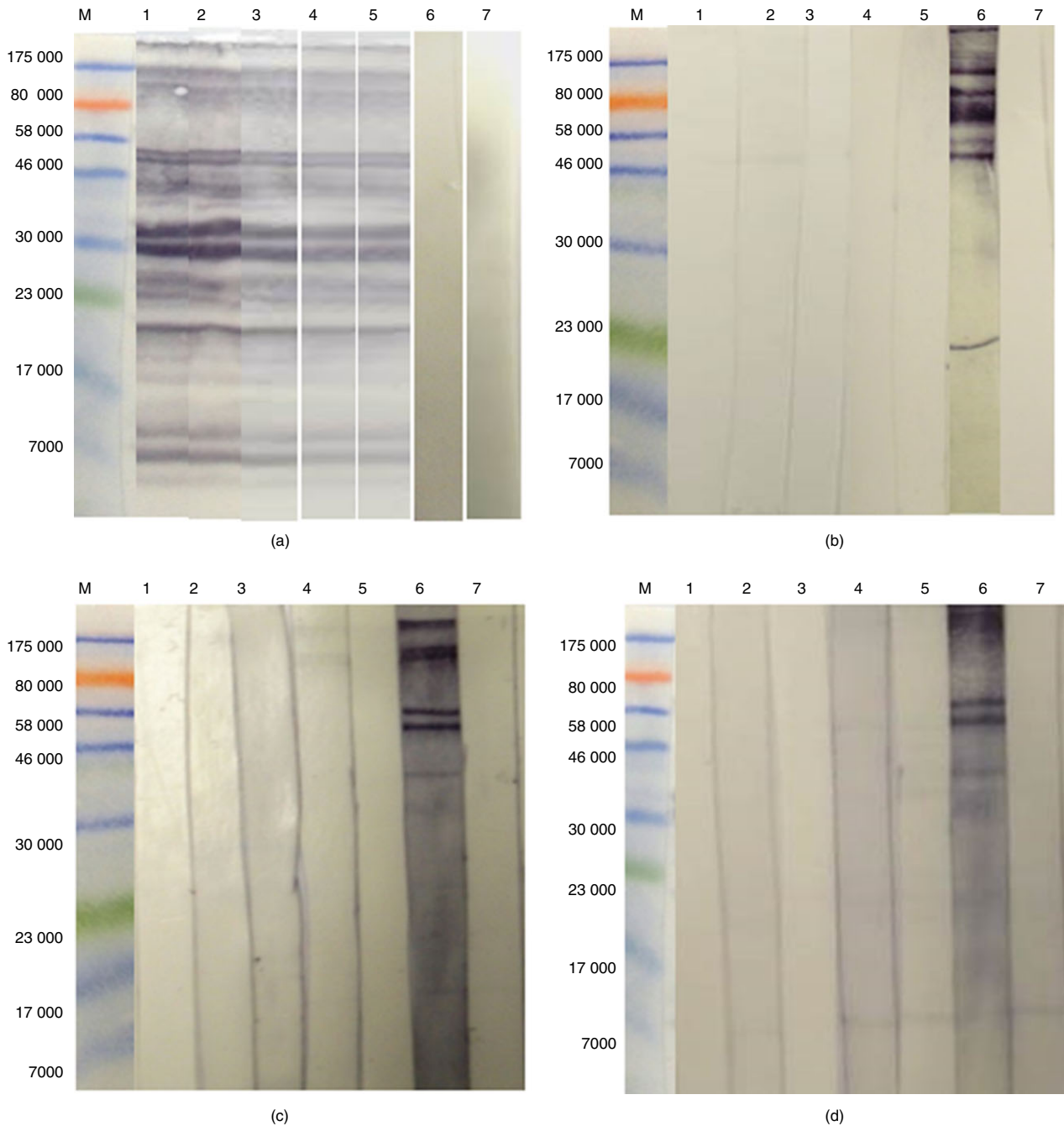


Figure 1. Western immunoblots of electrophoresed peanut and mouse tissue extracts probed with rabbit antisera. (a) Peanut, (b) mouse blood, (c) mouse kidney, (d) mouse spleen. (M) Molecular weight markers, (1) anti-*Schistosoma mansoni* soluble egg antigens (anti-SmSEA) serum, (2) anti-SmSEA serum, (3) anti-*S. mansoni* cercariae homogenate (anti-SmCH), (4) anti-SmCH, (5) anti-*S. mansoni* adult worm (anti-SmWh), (6) anti-normal mouse serum (anti-NMS), (7) anti-complete Freund's adjuvant (anti-CFA). Amounts of protein loaded per lane: 0.1 mg peanut extract; 0.003 mg NMS; 0.021 mg kidney extract; 0.214 mg spleen extract. [Colour figure can be viewed at wileyonlinelibrary.com]

were applied separately on immunoblotted constituents of aqueous extracts of mouse tissues (blood, kidney and liver, Fig. 1b–d, respectively). Results showed that none of the five anti-*S. mansoni* sera nor the anti-CFA serum demonstrated any reactivity with antigens in all three extracts from mouse (lanes 1, 2, 3, 4, 5, 7 in

Fig. 1b–d). Some antigens in each of the three extracts from mouse showed reactivity with antibodies in a rabbit anti-NMS that was used as a further control (lane 6 in Fig. 1b–d).

The anti-SmSEA antiserum used in lane 1, Fig. 1(a) was selected for use in subsequent experiments.

Two peanut antigens that reacted with the rabbit antibodies more conspicuously at ~30 000–33 000 MW in Fig. 1(a) were investigated further. Hence, Fig. 2(a) is a Western blot of peanut extract probed with the rabbit anti-SmSEA used in lane 1 in Fig. 1(a). Two arrows against the image of the Coomassie-stained SDS-PAGE gel in Fig. 2(b) point to two peanut molecules of ~30 000 and ~33 000 MW that were deemed to correspond to the most intensely reactive molecules in Fig. 2(a). Both of the peanut antigens of ~30 000–33 000 MW were purified from the gel and re-electrophoresis in SDS-PAGE to yield two seemingly distinct molecules (Fig. 2c).

The two purified peanut bands were subjected to MS analysis separately and data from each of the bands was searched against the NCBIInr database using the Mascot 'MSMS ions search' tool for peptide matching and protein identification. The results showed that each band had significant matches for two isoforms of the peanut allergen *Ara h 1*, namely clones P41B and P17, respectively. The MS data from both bands were therefore combined into a single Mascot search, which revealed a more significant match for *Ara h 1*, clones P41B (GI: 1168391; P43238.1) (see Supplementary material, Table S1) and P17 (GI: 1168390; P43237.1) (see Supplementary material, Table S2), respectively.

Both *Ara h 1* molecules, i.e. the ~30 000–33 000 MW pair of peanut antigens, were purified, electrophoresed in SDS-PAGE and electro-transferred onto nitrocellulose

paper (NCP). The NCP was probed in succession with a rabbit anti-SmSEA antiserum [the same as that used in Fig. 1(a), lane 1] and goat anti-rabbit IgG as the primary and secondary antibodies, respectively. A horizontal thin strip of NCP, corresponding to the area where the cross-reactive anti-SmSEA IgG antibodies bound to the purified ~30 000–33 000 MW peanut doublet antigens (i.e. between the two horizontal arrows in the Supplementary material, Fig. S1), was removed and the primary rabbit IgG antibodies were eluted from the NCP by treatment with low pH buffer.

Investigating the cross-reactivity of the eluted anti-SmSEA IgG antibodies

The rabbit anti-SmSEA IgG antibodies that were cross-reactive with *Ara h 1* and that had been purified from Western immunoblots by acid-elution were tested for reactivity on the constituents of unfractionated peanut extract, as well as on extracts of three mouse tissues, namely blood, kidney and spleen.

The results in Fig. 3(a) show that the eluted antibodies were, as expected, reactive with the peanut antigens from which they had been eluted, but also with many other antigens in the peanut extract (Fig. 3a, lane 1). A replicate NCP carrying peanut antigens, one incubated with antibody-acid-elution buffer alone and another with a rabbit anti-NMS, showed no reactivity (Fig. 3a, lanes 2

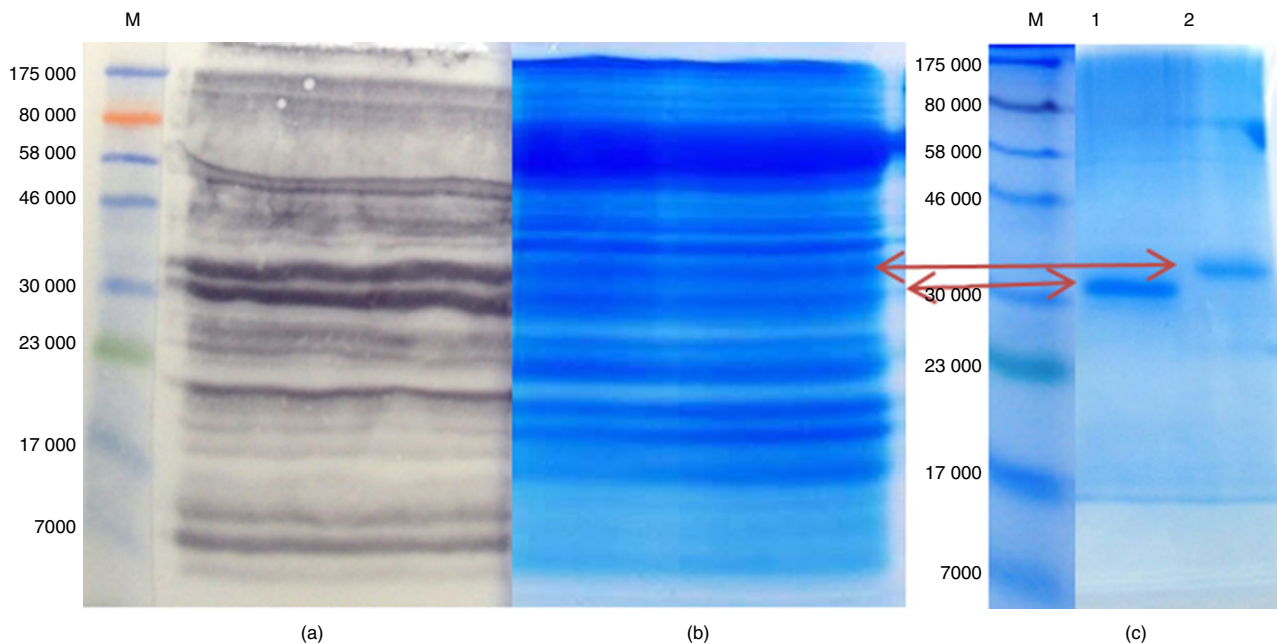


Figure 2. Steps showing the identification and purification of two cross-reactive peanut antigens. (a) Western immunoblot showing the identification of peanut antigens that are cross-reactive with IgG antibodies in an anti-*Schistosoma mansoni* soluble egg antigen (anti-SmSEA) serum, (b) a portion of gel "a" stained in Coomassie blue for identification and excision of a pair of cross-reactive peanut gel bands at ~30 000–33 000 MW intended for purification, (c) Coomassie-stained SDS-PAGE showing further resolution of the pair of peanut bands into a lower band (lane 1) and a top band (lane 2) for mass spectrometry analysis. [Colour figure can be viewed at wileyonlinelibrary.com]

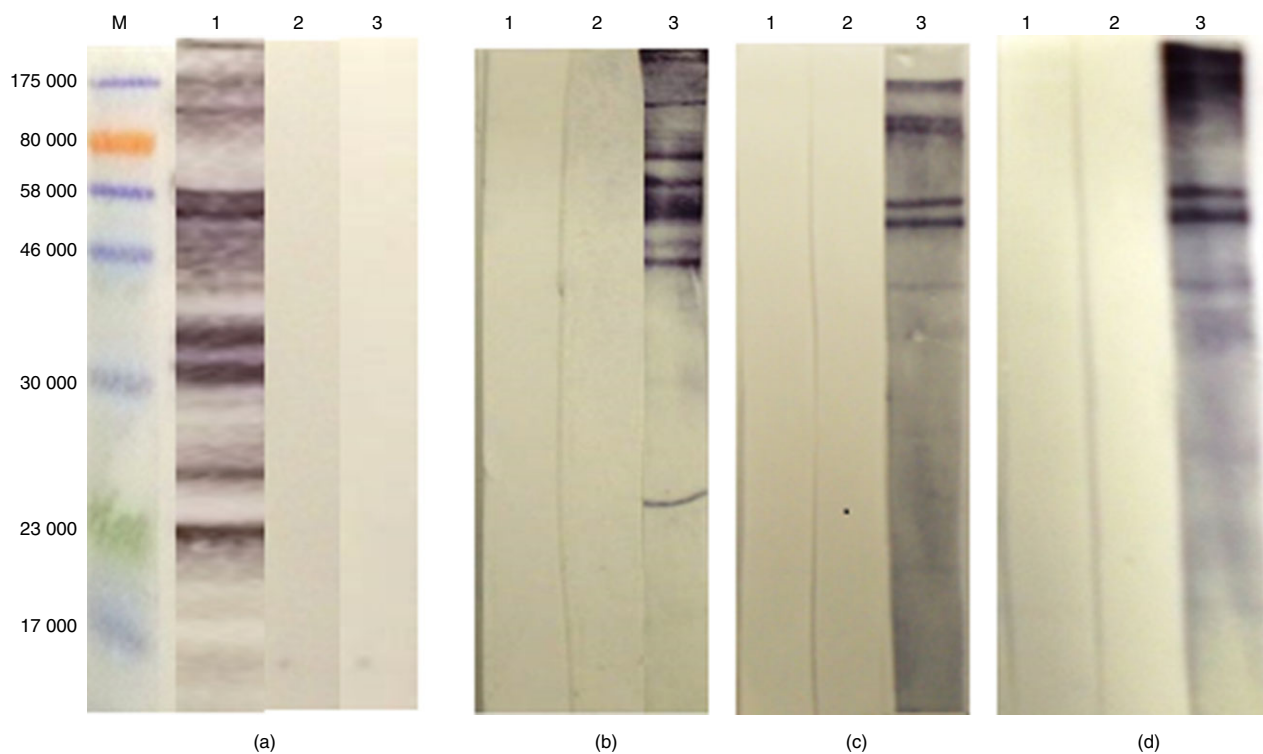


Figure 3. Western immunoblots probing extracts of peanut and mouse tissues with anti-*Schistosoma mansoni* soluble egg antigen (anti-SmSEA) IgG antibodies eluted by low pH buffer from a ~30 000–33 000 MW pair of peanut antigens. (M) Molecular weight marker. (a) Peanut, (b) normal mouse serum (NMS), (c) kidney, (d) spleen. Each strip was probed with: (1) eluted anti-SmSEA IgG antibodies, (2) incubated only in 1 ml acid-elution buffer, (3) rabbit anti-NMS. [Colour figure can be viewed at wileyonlinelibrary.com]

and 3, respectively). Furthermore, the eluted antibodies showed no reactivity against any of the three extracts from mouse tissue (lane 1 on Fig. 3b–d) and there was no reactivity after incubation in acid-elution buffer alone (lane 2, Fig. 3b–d). In contrast, some antigens of mainly high molecular weights in each of the three extracts from mouse showed reactivity with a rabbit anti-NMS serum (lane 3, Fig. 3b–d).

Tentative identification of *S. mansoni* egg antigens that induced the IgG antibodies in anti-SmSEA that cross-reacted with *Ara h 1* and other peanut antigens

To identify the *S. mansoni* egg antigen(s) that potentially induced the cross-reactive IgG antibodies in anti-SmSEA, the antibodies eluted from the ~30 000–33 000 MW peanut molecules were used to probe NCP carrying electro-transferred SmSEA in a Western immunoblot. Results in Fig. 4(a) show that the eluted IgG antibodies were reactive with two egg antigens; a broad band of ~36 000 and a band at ~100 000 MW. Based on previous experience of Western blot reactivity of the constituents of SmSEA, these two antigens were deemed likely to be IPSE/ α -1 and κ -5, respectively. Support for this conclusion was obtained by showing that the purified antibodies reacted seemingly with the same two antigens in SmSEA (Fig. 4a,

lane 1) as did two sera from rabbits immunized respectively with IPSE/ α -1 and κ -5 (Fig. 4a, lanes 2 and 3). Acid-elution buffer alone gave no reactivity against SmSEA (Fig. 4a, lane 4).

To confirm the identity of the two *S. mansoni* egg antigens putatively identified as IPSE/ α -1 and κ -5 respectively in Fig. 4(a), purification of the ~36 000 MW and the ~100 000 MW antigenic bands was undertaken by excision of bands corresponding to each separately from SDS-PAGE gels that had been loaded with SmSEA and elution of protein therefrom. Figure 4(b) shows a stained SDS-PAGE gel revealing the two purified egg antigens, of which the ~36 000 band in lane 1 was analysed in tandem MS analysis and its identity was confirmed as the IL-4-inducing protein precursor in *S. mansoni* (GI: 288948570), also known as IPSE/ α -1 (see Supplementary material, Table S3). Thereafter, the two purified antigens were electrophoresed separately and probed with the anti-SmSEA IgG antibodies eluted from the ~30 000–33 000 MW peanut moiety, two antisera monospecific for each of the respective egg antigens as well as a crude rabbit anti-SmSEA antiserum. Results in Fig. 4(c) show that the acid-eluted antibodies and monospecific rabbit anti-IPSE/ α -1 antibodies were reactive with purified ~36 000 MW egg antigen and likewise, the eluted antibodies also reacted with the purified ~100 000 MW egg antigen as

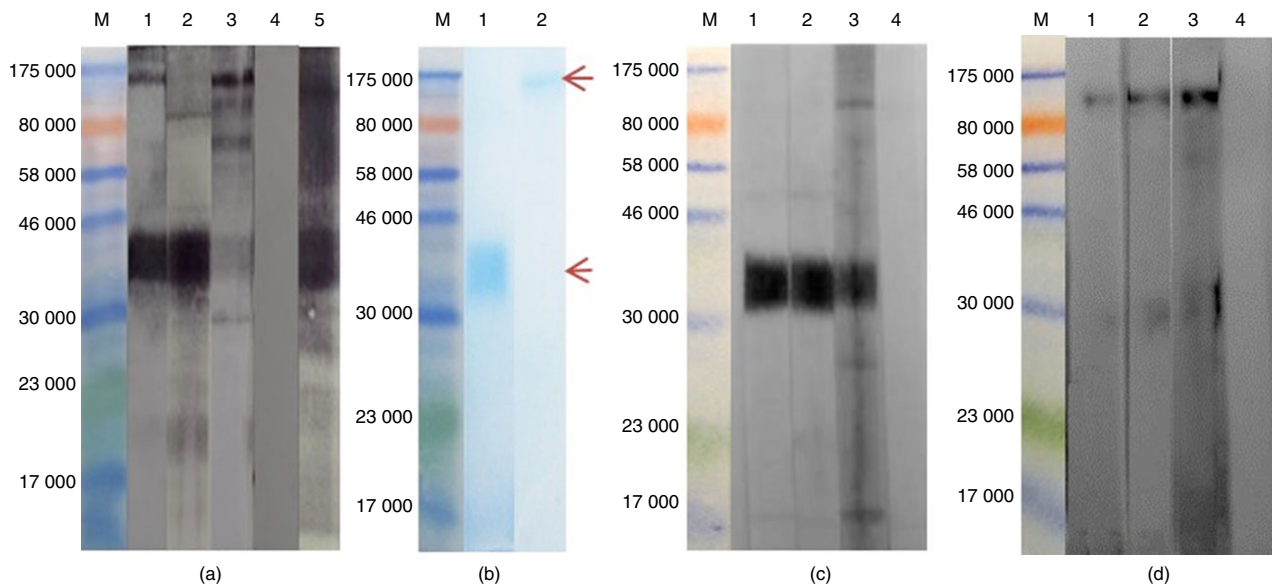


Figure 4. Western immunoblot probing *Schistosoma mansoni* soluble egg antigens (SmSEA) and two purified egg antigens with eluted anti-SmSEA IgG antibody to identify cross-reactive egg antigens. (M) Molecular weight markers. (a) SmSEA probed with; (1) eluted anti-SmSEA IgG antibodies, (2) rabbit anti-IPSE/α-1, (3) rabbit anti-κ-5, (4) incubated only in elution buffer, (5) rabbit anti-SmSEA. (b) SDS-PAGE showing the purified egg antigens after staining in SimplyBlue SafeStain; (1) IPSE/α-1, (2) κ-5. (c) Purified IPSE/α-1 probed with; (1) eluted anti-SmSEA IgG antibodies, (2) rabbit anti-IPSE/α-1, (3) rabbit anti-SmSEA, (4) incubated only in elution buffer. (d) purified κ-5 probed with; (1) eluted anti-SmSEA IgG antibodies, (2) rabbit anti-κ-5, (3) rabbit anti-SmSEA, (4) incubated only in elution buffer. [Colour figure can be viewed at wileyonlinelibrary.com]

did the monospecific anti-κ-5 (Fig. 4d). Untreated rabbit anti-SmSEA antiserum had antibodies that reacted against both antigens, seemingly IPSE/α-1 and κ-5 (lanes 3 in Fig. 4c,d).

Investigating peptide identity between cross-reactive *S. mansoni* antigens and *Ara h 1*

Following the identification of IPSE/α-1 and κ-5 as potential inducers of anti-SmSEA IgG antibodies that are cross-reactive with *Ara h 1*, the amino acid sequences of the two egg antigens; [IPSE/α-1 (GI: 28894857, AAK26170.1) and κ-5 (GI: 62462031, AAX83114.1)], respectively, were aligned with that of *Ara h 1* (GI: 1168391; P43238.1) using the pairwise sequence alignment tool from the EMBL-EBI database to ascertain the level of peptide identity. Results showed a peptide identity of 3.5% between *Ara h 1* and IPSE/α-1 (see Supplementary material, Table S4), while the alignment of *Ara h 1* and κ-5 revealed an identity of 9.8% (see Supplementary material, Table S5). All these results reflect a low level of protein sequence identity.

The cross-reactivity of the eluted anti-SmSEA IgG antibodies with molecules in other plant extracts and the possible involvement of CCDs

Due to the observed cross-reactivity of the eluted anti-SmSEA IgG antibodies with *Ara h 1* as well as

with other peanut molecules, their reactivity with constituents of extracts of six other plants with the potential to cause allergy in humans; latex, banana, tomato, melon, avocado, and kiwi, was investigated by Western blotting. Results in Fig. 5(a) showed that the purified antibodies were cross-reactive with an array of molecules in extracts of the six constituents of plants as well as the two egg antigens in SmSEA already identified above as IPSE/α-1 and κ-5, respectively.

As only a low level of amino acid-sequence identity was found between IPSE/α-1 and κ-5, and *Ara h 1* (see Supplementary material, Tables S4 and S5 respectively), an alternative possible explanation for the antigenic cross-reactivity was investigated in terms of shared carbohydrate determinants (CCDs) by means of treatment with mild sodium metaperiodate. Treatment of the replicate NCP in Fig. 5(a) carrying electroblotted proteins with a solution of 20 mM sodium metaperiodate dissolved in sodium acetate buffer, pH 4.5, before probing with the purified antibodies, resulted in disappearance of all antigenic reactivity in lanes 1–8 of Fig. 5(b). Only the broad ~36 000 MW band in SmSEA, identified above as IPSE/α-1, retained antigenic reactivity (Fig. 5b, lane 9).

This observation is an indication that the antigenic cross-reactivity demonstrated here is due to a widespread distribution of CCDs on the antigenically cross-reactive molecules in these extracts.

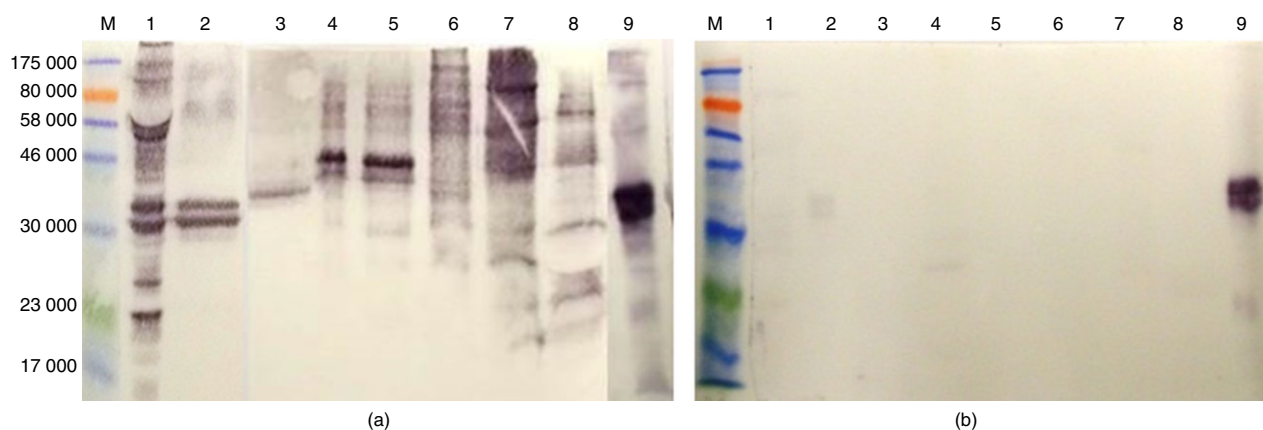


Figure 5. Constituents of different plants probed with the acid-eluted anti-*Schistosoma mansoni* soluble egg antigen (anti-SmSEA) IgG antibodies and the effect of treatment with periodate. (a) Incubated in 50 mM sodium acetate buffer before probing with eluted anti-SmSEA IgG antibodies, (b) Incubated in 20 mM sodium metaperiodate dissolved in 50 mM sodium acetate buffer before probing with the same primary antibody as 'a'. (M) Molecular weight marker, (1) unfractionated peanut, (2) purified pair of peanut antigens at ~30 000–33 000 MW, (3) crude latex (0.2 mg), (4) banana (0.16 mg), (5) tomato (0.27 mg), (6) melon (0.24 mg), (7) avocado (0.22 mg), (8) kiwi (0.19 mg), (9) SmSEA (0.010 mg). [Colour figure can be viewed at wileyonlinelibrary.com]

Predicting potential glycosylation sites on cross-reactive *S. mansoni* antigens and *Ara h 1*

Consequent upon the observations in Fig. 5 and the Supplementary material (Tables S4 and S5, respectively), the amino acid sequence of *Ara h 1* (GI: 1168391; P43238.1) was searched against the GlycoEP software for the prediction of potential *N*- and/or *O*-linked glycosylation sites.⁴³ Results revealed that *Ara h 1* has one potential *N*-linked glycosylation site at position N-521 (shown in underlined and italicized font in the Supplementary material, Table S6), and eight potential *O*-linked glycosylation sites (at positions T-23, 34, 81, 82, 184 and 246 and S-170 and 523, shown in underlined and italicized font in the Supplementary material, Table S7). Also, a search of *Ara h 1* against the ExPASy software (Bioinformatics Resource Portal) using the GLYCOMOD prediction tool confirmed that it has one *N*-linked potential glycosylation site at the same position, N-521, as indicated by the GLYCOEP software (result not shown).

Discussion

We have previously demonstrated that rabbit anti-schistosome IgG antibodies cross-react with many different constituents of plants and invertebrates that are allergic to humans.²⁸ The cross-reactivity between *S. mansoni* egg and cercarial antigens and a 43 000 MW molecule in natural rubber latex was investigated in detail and the latter was found to be latex allergen *Hev b 7*.

Allergy to latex is now less important so the methods used in that study were here extended to investigate the extent to which they might be applied to another allergen, namely peanut, allergy to which is currently of more significance than that to latex and the constituents of

which were also shown to be antigenically cross-reactive with *S. mansoni*.²⁸

Human allergic sensitization to peanut is widespread in the western/developed world.³¹ Conversely, in developing and underdeveloped countries where the prevalence of *S. mansoni* and other helminths is higher, incidence of peanut and other food allergies is relatively low and a low prevalence of skin-prick positivity to peanut has been observed in persons with schistosome infections who are resident in a parasite-endemic region of Ghana, despite their having anti-peanut IgE antibodies.¹⁵ This observation is consistent with the tenets of the hygiene hypothesis. Results in Fig. 1 confirm the earlier indications²⁸ that extensive IgG cross-reactivity occurs between *S. mansoni* and peanut antigens/allergens. The non-reactivity of the anti-SmSEA antibodies against any of the antigens/molecules in the three tissue extracts from mouse is an indication that the cross-reactivity of the purified antibodies with antigens in extracts of peanut and those in other plants with potential to cause allergy in humans is not a ubiquitous property of rabbit sera in general.

The pattern of cross-reactivity that drew our particular attention was that against a pair of bands at ~30 000–33 000 MW in the peanut extract, which reacted somewhat more intensely than other peanut antigens with antibodies from the two rabbit anti-*S. mansoni* egg antisera. Subsequent investigations used one of these rabbit anti-SmSEA sera as it had previously been observed to be cross-reactive with antigens in various plant and invertebrate extracts.²⁸

The two peanut proteins that reacted with the rabbit anti-SmSEA antibodies were purified and MS analysis identified both as the peanut allergen, *Ara h 1*. *Ara h 1* is a glycoprotein monomer usually found to have an

approximate molecular mass of 63 000 in SDS-PAGE and IgE immunoblots.^{47–49} However, in this study, two lower molecular weight bands, both containing *Ara h 1* peptides, were observed at ~30 000–33 000 MW. It is possible that the initial protein mass of ~63 000 was broken down/reduced during sample extraction and processing by SDS, as the 30 000 and 33 000 'bands' observed herein together would add up to about the correct mass of *Ara h 1*. Hence, the discrepancy with conventional estimates of the molecular mass of *Ara h 1* could be due to the existence of various isoforms of the allergen, with a minor form having been previously reported to occur as a 33 000 MW band.⁵⁰

Ara h 1 is one of the most important peanut allergens,⁴⁸ as it is recognized by IgE antibodies in approximately 94% of sera from patients with peanut allergy.⁵¹

Purified rabbit anti-SmSEA IgG antibodies that bound to the purified ~30 000–33 000 peanut doublet containing *Ara h 1* on NCP and that were eluted by low pH treatment therefrom helped identify two *S. mansoni* egg antigens that may have induced these cross-reactive antibodies, namely IPSE/ α -1⁵² and κ -5,⁵³ respectively. This conclusion is based on the observations in Fig. 4(a, c, d) that the anti-SmSEA antibodies eluted from the ~30 000–33 000 peanut antigens reacted with the two respective antigens in SmSEA, similarly to two rabbit antisera known to contain antibodies monospecific for IPSE/ α -1 and κ -5, respectively. Moreover, MS analysis of the purified ~36 000 antigen in SmSEA confirmed its identity as IPSE/ α -1.

IPSE/ α -1 has been identified as a factor in SmSEA capable of inducing the release of IL-4 from basophils by interacting with IgE on the surface of the cells,⁵⁴ albeit not in an antigenically specific manner.^{55,56} Further reports on IPSE/ α -1 indicate that a nuclear localization signal is present in its amino acid sequence.⁵⁷ IPSE/ α -1 expression has been reported to be restricted to the egg stage of *S. mansoni*⁵² and the molecule has recently been identified as belonging to the protein superfamily of $\beta\gamma$ crystallins.⁵⁵ The molecule also binds IgG to some extent, also in a 'non-specific' manner (i.e. by a mechanism that does not involve the immunoglobulin's antigen-binding site), but with a fourfold lower affinity than IgE.⁵⁵ This immunologically non-specific interaction has previously been shown not to account for the interaction of the rabbit IgG antibodies with IPSE/ α -1,²⁸ and in this study also it can be seen that IgG in the anti- κ -5 antiserum used in Fig. 4a, lane 3, reacted much less intensely with IPSE/ α -1 than anti-IPSE/ α -1 IgG antibodies.

κ -5 is another immunogenic glycoprotein in SmSEA and it reacts with both IgG and IgE isotype antibodies in sera from individuals with *S. mansoni* infection.⁵³ Furthermore, the κ -5-specific IgE antibodies in sera of *S. mansoni*-infected individuals were directed against the

core region of the κ -5 glycans.⁵⁸ The biological role of κ -5 is not yet known.

Attempts to detect peptide similarity between *Ara h 1* and the two cross-reactive *S. mansoni* egg antigens (IPSE/ α -1 and κ -5) revealed a low level of identity that is probably insufficient for the cross-reactivity to be due solely to peptide epitopes (see Supplementary material, Tables S4 and S5). The findings herein are further corroborated by a report⁵³ that the sequence of κ -5 shows significant similarity only with an uncharacterized mRNA in *S. japonicum*. Neither IPSE/ α -1 nor κ -5 have been shown to have allergenic properties, though *Ara h 1*, a glycoprotein belonging to the vicilin-seed storage protein family of the 7S globulins,^{59–62} has well-defined allergenicity.

The proteinaceous properties of the three molecules therefore seem unlikely to be responsible for the antigenic *S. mansoni*/peanut cross-reactivity, reports on similarities in protein families between allergens and some helminth proteins notwithstanding.^{63–65}

Anti-SmSEA IgG antibodies eluted from peanut allergen *Ara h 1* cross-reacted not only with peanut *Ara h 1*, but also with the constituents of six other plants (Fig. 5a). It is also noteworthy that the anti-SmSEA antibodies that had been eluted from *Ara h 1* cross-reacted with many other constituents of the peanut extract (Figs 4 and 5a). These observations are remarkable and may be due to the presence of shared epitopes on the cross-reactive molecules in peanut as well as on constituents of the six other plant extracts.

Previously, cross-reactivity among plant allergens and with some molecules in invertebrates including helminths, have been reported and suspected to be due to shared CCDs.^{66–68} *N*-linked glycans containing core fucose and xylose are known to be commonly distributed among different organisms and have been implicated in IgE cross-reactivity between plants and invertebrates.^{69,70} Hence, based on the limited evidence for any involvement of shared peptide epitopes outlined above, investigations on the possible involvement of CCDs in the antigenic cross-reactivity were initiated. NCP carrying constituents from six different plants were treated with mild sodium metaperiodate before probing with the acid-eluted anti-SmSEA IgG antibodies. The treatment abrogated virtually all of the cross-reactivity against constituents of the plant extracts (Fig. 5b), indicating an involvement of glycan epitopes that are common to both the *S. mansoni* egg antigens and the peanut allergen *Ara h 1*, though reactivity against IPSE/ α -1 survived the chemical treatment (Fig. 5b, lane 9).

The reason for the inability of sodium metaperiodate to destroy the reactivity of IPSE/ α -1 with the acid-eluted anti-*Ara h 1* antibodies remains an enigma, although similar findings with respect to this molecule have been previously reported.^{28,45} Anti-CCD antibodies induced by horseradish peroxidase in mice and cross-reactive with

phytohaemagglutinin also showed resistance to periodate treatment and it was suggested that horseradish peroxidase-induced anti-CCD antibodies were reacting with the trisaccharide backbone of the molecule, rather than the periodate-degradable glycan residues containing xylose, fucose or mannose.⁷¹

IPSE/ α -1 and κ -5 are both glycosylated egg antigens with well-characterized glycan structures.^{58,72,73} Each peptide of the IPSE/ α -1 dimer contains two *N*-linked glycosylation sites, which are fucosylated, in addition to an immunogenic Lewis-X terminus,⁷² while κ -5 has four occupied *N*-linked glycans with attached fucose and xylose residues in addition to an immunogenic LDN (GalNAc β 1-4GlcNAc) residue.⁵⁸ Previous reports on *Ara h 1* showed that it possesses one *N*-linked glycan with attached xylose and mannose residues.⁷⁴ Prediction of potential glycosylation sites on the amino acid sequence of *Ara h 1* using recently published software⁴³ also revealed it to possess one potentially *N*-linked and eight *O*-linked glycosylation sites (see Supplementary material, Tables S6 and S7, respectively) and prediction using the ExPASy software (GlycoMod) concurred with the above findings. The presence of identical immunogenic glycan structures on the two *S. mansoni* egg antigens and *Ara h 1* could account for the observed cross-reactivity of the rabbit anti-SmSEA IgG antibodies.

To summarize, this study has shown antigenic cross-reactivity between the peanut allergen *Ara h 1* and, putatively, two *S. mansoni* egg antigens; IPSE/ α -1 and κ -5, as well as with some other antigenic constituents of six plants associated with human allergies. Peptide identities between the two egg antigens and *Ara h 1* were not significant and involvement of CCDs has been implicated on the basis that most of the cross-reactivity was periodate-sensitive, the antigenicity of IPSE/ α -1 being an exception to this sensitivity. A recent report has indicated that 'schistosome infection-induced IgE against CCDs might account largely for high IgE levels to peanut in a population of Ghanaian schoolchildren... (though)... no evidence of IgE-mediated peanut allergy was found'.¹⁵

Findings herein are novel and could offer a possible explanation for the hygiene hypothesis. Glycans in schistosomes and allergens have been shown to drive Th2 immune responsiveness and through the induction of regulatory T cells have been implicated in subsequent immunomodulation of this response in hosts infected with the parasite.⁸ The consequences of the immunoregulation in turn may have an effect dampening Th2-driven allergic responses.¹¹ Alternatively, IgG antibodies induced by the parasitic infection and which cross-react with plant- and invertebrate-derived CCDs may inhibit the reactivity of anti-allergen IgE antibodies;⁷⁵ i.e. in schistosome-endemic areas, the inverse association observed between infection with the parasite and allergies could be due to the anti-schistosome CCD IgG in some way

blocking the activity of allergen-specific IgE, thereby preventing allergic reactivity. In this context, allergen-specific immunotherapy is commonly found to induce production of anti-allergen IgG antibodies, particularly of the IgG4 subclass, and these have been shown to block *in vitro* reactions mediated by allergen-specific IgE.^{22,76,77} IgG antibodies induced by helminth infection may have a similar effect.

Thus, schistosome-induced IgG antibodies that are cross-reactive with allergens such as *Ara h 1* may block allergic hypersensitivity reactions, and so offer an explanation for the relative absence of peanut allergy in schistosome-infected children in Ghana.¹⁵ Allergen-specific immunotherapy for peanut allergy is having some, but not great, success³¹ and further work to investigate whether IgG antibodies induced by schistosome infection can more effectively block the reactivity of IgE antibodies reactive against peanut and other allergens, which have CCDs in common is warranted.

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Disclosures

The authors declare no conflict of interest.

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blocking IgG toward IgE epitopes and shifts immune responses to a tolerogenic and Th1 phenotype. *J Immunol* 2013; **190**:3068–78.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Steps illustrating the methods of purification by acid-elution of anti-*Schistosoma mansoni* soluble egg antigens (anti-SmSEA) IgG antibodies that are cross-reactive with the pair of peanut *Ara h 1* bands at ~30 000–33 000 MW. Cross-reactive anti-SmSEA IgG antibodies were eluted from cut-out nitrocellulose paper strip as shown in the marked area between two horizontal arrows using elution buffer.

Table S1. MASCOT search output of tandem mass spectrometry data from the purified pair of peanut gel bands at ~30 000–33 000 MW and matching allergen *Ara h 1*, clone P41B.

Table S2. Mass spectrometry-derived data matching *Ara h 1*, clone P17.

Table S3. MASCOT search output of tandem MS data from the purified ~36 000 MW gel band in *Schistosoma mansoni* soluble egg antigens.

Table S4. Alignment of the amino acid sequences of IPSE/ α -1 and *Ara h 1*.

Table S5. Alignment of the amino acid sequences of κ -5 and *Ara h 1*.

Table S6. Predicting potential N-linked glycosylation sites for *Ara h 1*.

Table S7. Predicting potential O-linked glycosylation sites for *Ara h 1*.