

An Immunochemical Investigation of the Allergens from *Ascaris suum* Perienteric Fluid

Cross-Reactivity, Molecular Weight Distribution and Correlation with Phosphorylcholine-Containing Components

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Abstract. An extract of *Ascaris suum* perienteric fluid was fractionated on Sephadex G-200 and the resulting high molecular weight (approx. 600,000 daltons) and low molecular weight (approx. 16,000 daltons) fractions examined for allergenicity using human sera. Radioallergosorbent (RAST) and RAST-inhibition assays showed that the high molecular weight fraction was 15- to 40-fold more potent than the low molecular weight fraction. Crossed radio-immunoelectrophoretic analysis showed that the high molecular weight fraction contained 10 allergens in contrast to the 6 comprising the low molecular weight fraction. Polyacrylamide gel electrophoretic studies in combination with RAST indicated that two high molecular weight glycoprotein-staining components may be major allergens. *A. suum* cross-reacted allergenically with *Toxocara canis* and various life cycle stages of *Nippostrongylus brasiliensis* but not with *Strongyloides ratti* or *Schistosoma mattheei*. The cross-reacting allergens were predominantly of high molecular weight and the molecular weight distribution pattern obtained for *A. suum*, *T. canis* and *N. brasiliensis* paralleled that obtained for the distribution of the carrier of the hapten phosphorylcholine (Pc). However, it was shown that the hapten per se was not allergenic. In contrast, analysis of the allergens from *N. brasiliensis* using sera from rats infected with the same parasite showed that the homologous allergens were predominantly of low molecular weight (approx. 20,000 daltons) and did not contain Pc.

Introduction

Many attempts have been made to isolate and characterize the major allergens from the closely related helminths *Ascaris lumbricoides* and *Ascaris suum* but in the majority of studies, the definition of allergenicity is based on data obtained from animal models rather than on those derived from human studies [reviewed in 8, 13, 17]. Allergenicity in these models is primarily associated with a few low molecular weight allergens (range 15,000-45,000 daltons) of restricted isoelectric point (pH 5-6), whereas when assayed against human sera [15, 23] allergens from *A. suum* demonstrate a wider range of both apparent molecular weight (14,000->360,000) and pI (pH 4-8).

It is likely that these contrasting findings result

from either differences in the spectrum of antigens exposed to the host or their manner of presentation. For example, animal models either employ homogenized extracts in adjuvant or larvae which do not develop into adulthood in these unnatural hosts. Humans infected with *A. lumbricoides*, in contrast, are exposed to antigens liberated from both larval and adult worms [8,13] and it is logical to assume that human sera might recognize a wider spectrum of allergens than that revealed in murine models.

On the basis of the above findings we have undertaken a systematic analysis of the allergens from *A. suum* using human sera, and in so doing have investigated the cross-reactivity of these allergens with those from other helminths. In addition, we have investigated the potential correlation between the allergenicity of parasite molecules and the presence of the

happen phosphorylcholine (Pc), since it has been shown that hosts infected with parasites containing Pc produce significant anti-Pc responses [6, 14].

Materials and Methods

Parasite Extracts

A. suum worms were collected at a local abattoir and washed in warm tap water. The posterior end (1 cm) of motile worms was removed and the perienteric fluid expressed. The fluid was filtered through a 0.4 μ m membrane filter (Sartorius GmbH, FRG) and dialyzed against 100 vol of either 0.05 M ammonium bicarbonate buffer, pH 8.0, or 0.01 M phosphate buffer, pH 7.2, at 4°C. Any precipitate resulting from dialysis was removed by centrifugation and the extract either lyophilized or stored at -20°C.

Toxocara canis worms were obtained from dogs on autopsy. *Nippostrongylus brasiliensis* worms, larvae and excretory/secretory products were provided by Dr. W. Allan, Clinical Immunology Research Unit, Perth. *Strongyloides ratti* L₃ larvae were provided by Dr. H. Dawkins, University of Western Australia. *Schistosoma mattheei* were provided by Dr. E. Fourie, University of Pretoria, S. Africa.

Soluble extracts of these parasites were prepared in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) using a Potter-Elvehjem tissue grinder. Insoluble material was removed by centrifugation and extracts stored at -20°C. *N. brasiliensis* larvae and excretory/secretory extracts were prepared as described previously [1].

Preparation of Pc-Bovine Serum Albumin Conjugate (Pc-BSA)

p-Diazonium phenylphosphorylcholine was prepared from *p*-nitrophenyl-phosphorylcholine (Biosearch, San Rafael, Calif., USA) and coupled to bovine serum albumin (BSA; Sigma Chemical Co. Ltd., St. Louis, Mo., USA) as described [5, 9]. The molar coupling ratio of Pc to BSA was shown to be 11:1 using a molar absorptivity of 12,600 at 475 nm in 0.1 N NaOH.

Analytical Methods

The protein and carbohydrate content of various fractions were determined using the Lowry modification of the Folin assay and the phenol-sulphuric acid assay respectively as described previously [21]. The primary standards used were BSA and *D*-glucose (BDH Ltd., Poole, UK).

Ascaris Allergic Sera

Sera were obtained from Papua New Guinean (PNG) donors and screened for *Ascaris*-specific IgE by radioallergosorbent (RAST) assay using discs optimally coated with *A. suum* extract. Sera producing high RAST (class 4) scores were pooled, aliquoted and stored at -20°C.

RAST Assays

The allergenicity of extracts and gel filtration fractions was determined as described previously [23] by either direct RAST assay or inhibition of RAST using CNBr-activated paper discs and ¹²⁵I-labelled anti-human IgE reagent (Pharmacia South Seas Pty. Ltd., Australia). The results obtained in both types of assay were analyzed

by linear regression analysis and only those sets of data giving correlation coefficients > 0.9 were used.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed as described previously [21]. The distribution of allergenicity of extracts after PAGE was performed by direct RAST assay. Gels were sliced into 1-mm sections and each section added to wells of a 96-well microtitre tray. After addition of 1 CNBr-activated paper disc and 100 μ l of 0.01 M sodium bicarbonate buffer, pH 8.0, the trays were incubated overnight. The discs were transferred to 5-ml plastic tubes and processed as for RAST.

Rabbit Anti-*A. suum* Antiserum

Rabbit anti-*A. suum* antiserum was prepared by immunizing rabbits intramuscularly with *A. suum* perienteric fluid extract (10 mg) emulsified in complete Freund's adjuvant. Secondary subcutaneous injections were performed 14 days later and blood collected 10 days after this and subsequent booster injections. Sera from different bleeds were pooled and stored at -20°C until required.

Crossed Immunoelectrophoresis (CIE)

CIE and crossed radioimmunoelectrophoretic (CRIE) analyses of *A. suum* and gel filtration fractions were performed as described previously [21] using 1% w/v agarose (Type V; Sigma Chemical Co.) and 20% v/v rabbit anti-*A. suum* perienteric fluid antiserum.

CRIE was performed using pooled PNG *Ascaris* allergic serum diluted 1:5 with PBS. After 48 h incubation at room temperature, plates were washed, pressed and incubated with ¹²⁵I-labelled rabbit anti-human IgE (Pharmacia RAST reagent, 300,000 cpm/plate) diluted in PBS containing 0.3% v/v Tween 20. After overnight incubation, the plates were washed in distilled water and dried. Autoradiography was performed using Kodak Ortho film for 14 days and the film processed in an automatic processor. The CIE plates were stained and then photographed. A negative *A. suum* RAST serum pool was also studied in parallel.

Detection of Pc-Containing and Con A-Reactive Components

The presence of Pc and Con A-reactive components were determined by inhibition of haemagglutination (HAI) assays as described previously [20]. Results were expressed as the reciprocal of the highest dilution of material producing inhibition.

Gel-Filtration Studies

The parasite extracts were fractionated by upward elution at 20 ml/h either on a column (81 $\times$ 2.6 cm) of Sephacryl S-300 or a column (100 $\times$ 5 cm) of Sephadex G-200 (Pharmacia South Seas Pty. Ltd.) equilibrated with 0.05 M ammonium bicarbonate buffer, pH 8.0.

Protein elution profiles were determined by measuring the absorption at 280 nm and appropriate fractions were pooled and lyophilized. The apparent molecular weight distribution of components was obtained by reference to a standard curve of K_{av} versus log molecular weight. The apparent molecular weight markers were obtained from Pharmacia and included thyroglobulin (MW 669,000 daltons), ferritin (440,000), catalase (232,000), ovalbumin (43,000), chymotrypsinogen (25,700) and ribonuclease (13,700). Data were analyzed by linear regression.

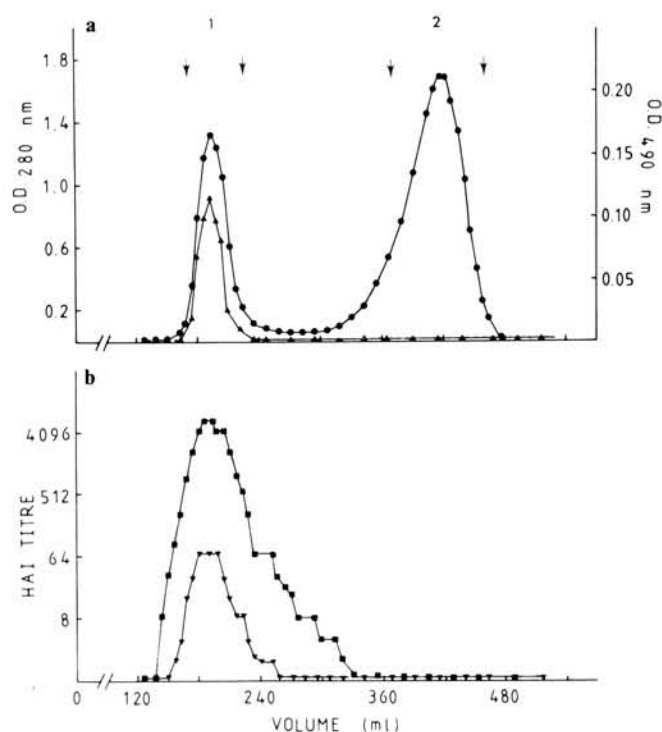


Fig. 1. Sephadex G-200 chromatographic elution profile of *A. suum* perienteric fluid (50 ml). Fractions were analyzed for protein by absorbance at 280 nm (●) and for carbohydrate (▲) using the phenol-sulphuric acid assay (a). Fractions were also analyzed for Con A reactivity (▼) and Pc content (■) by HAI (b).

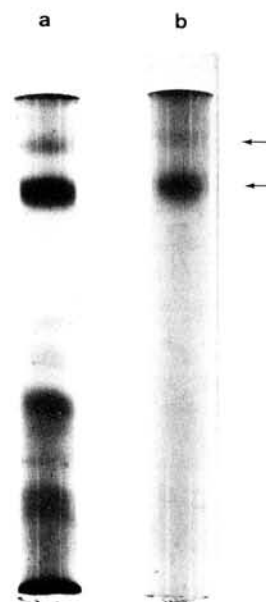


Fig. 2. 5% polyacrylamide gel electrophoretic analysis of *A. suum* perienteric fluid (200 μg). Gels were stained for protein with Biebrich scarlet (a) and for carbohydrate with periodic acid-Schiff's reagent (b). The arrows indicate the two prominent cathodic glycoproteins.

N. brasiliensis Allergens

The molecular weight distribution of allergens isolated by gel filtration of *N. brasiliensis* extracts was determined using a modification of the Prausnitz-Kustner (PK) assay. Aliquots (50 μl) of that dilution of serum from *N. brasiliensis*-infected Sprague-Dawley (SD) rats which produced 50% of maximum reaction in a passive cutaneous anaphylaxis assay were injected intradermally into the shaved backs of uninfected SD rats. Twenty-four hours later, the animals were injected intravenously with 800 μl of 1% w/v Evans blue in Tyrode's solution. Twenty minutes later, 50 μl of each gel filtration fraction was injected intradermally into the same site as the serum. The reaction was allowed to proceed for 20 min and the diameter of the bluing reaction determined. The data obtained were corrected for nonspecific bluing using results obtained from parallel experiments performed with a control serum.

Results

Physicochemical Analysis of *A. suum* Perienteric Fluid

A. suum perienteric fluid was fractionated on Sephadex G-200 to produce two major elution peaks (fig. 1a), one of high molecular weight (approx.

600,000 daltons) and the other of low molecular weight (approx. 16,000 daltons). Analysis of individual elution fractions showed that carbohydrate, Con A-reactive sugar residues, and Pc content (fig. 1b) were confined to the high molecular weight peak. Fractions comprising these two peaks were pooled (designated HMWP and LMWP) and analyzed further. PAGE analysis showed that whilst HMWP was heterogeneous with regard to protein, only 2 components, migrating in the top portion of the gel, appeared to be glycoprotein (fig. 2).

Determination of Allergenicity

A comparison of direct RAST activities (table I) showed that 50% maximum RAST binding was achieved by 0.75 μg/ml of perienteric fluid, 0.05 μg/ml HMWP and 28 μg/ml LMWP. In contrast, perienteric fluid was the most potent preparation when analyzed by RAST inhibition (table I), requiring 91 μg/ml compared with 121 μg/ml for HMWP and 2,480 μg/ml for LMWP to achieve 50% inhibition.

Table I. RAST and RAST inhibition analyses of *A. suum* perienteric fluid and the Sephadex G-200 HMWP and LMWP

Fraction	Amount of material (in $\mu\text{g/ml}$) required to produce 50% of maximum RAST binding	Relative potency ¹	Amount of material (in $\mu\text{g/ml}$) required to produce a 50% inhibition of RAST binding ²	Relative potency ¹
<i>A. suum</i> perienteric fluid	0.75	100	91	100
HMWP	0.05	1,500	121	75
LMWP	28	2.6	2,481	3.6

¹ Relative potency was expressed as a percentage of the *A. suum* perienteric fluid.

² Discs were coated with *A. suum* perienteric fluid.

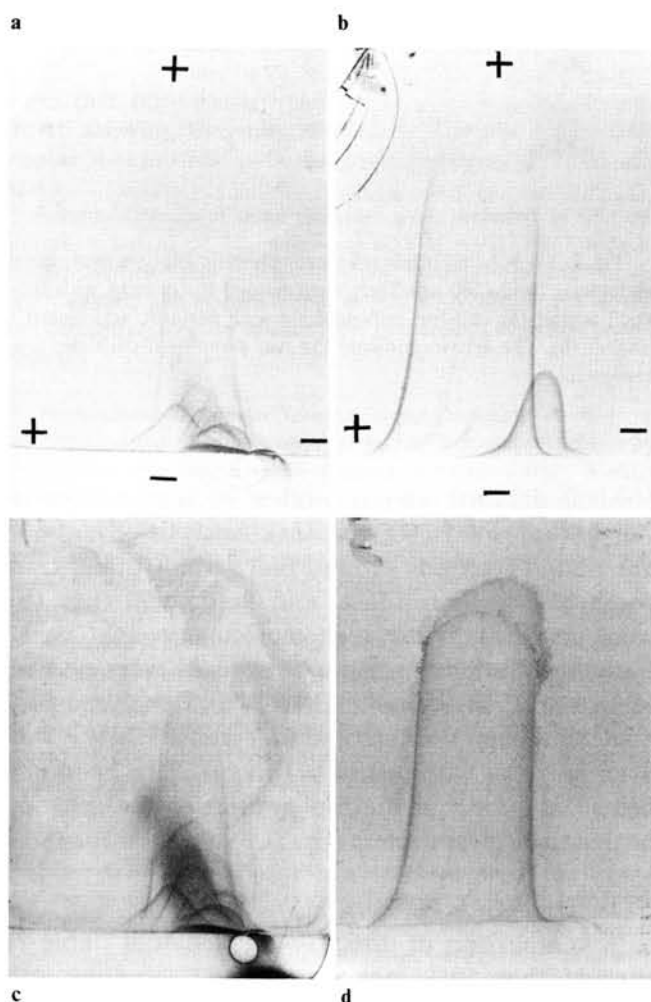


Fig. 3. CIE and CRIE analyses of the Sephadex G-200 gel filtration fractions of *A. suum* perienteric fluid using 20% v/v anti-*A. suum* perienteric fluid antiserum and *Ascaris* RAST-positive sera from PNG donors. CIE (a) and CRIE (c) of the high molecular weight fraction, and CIE (b) and CRIE (d) of the low molecular weight fraction.

The antigenic and allergenic content of the HMWP and LMWP were examined by CIE and CRIE, the results of which are shown in figure 3a-d. *A. suum* contained approximately 17 antigens, most of which appeared to be allergens (data not shown). Similarly, all of the 10 antigens identified in HMWP bound IgE from pooled *A. suum* allergic sera and hence were deemed allergens (fig. 3a, c). LMWP was shown to contain 4 antigens but CRIE revealed that there were 6 IgE-binding antigens (fig. 3a, d). Fused rocket immuno-electrophoresis revealed (data not shown) that there was little or no overlap of components in HMWP and LMWP.

The allergenic profile of material fractionated by PAGE was determined by RAST-binding analysis on material eluted from 1-mm slices of appropriate gels. After electrophoresis, *A. suum* perienteric showed high RAST-binding activity throughout the gel (7–21% radiation bound) although the majority was confined to the cathodic half. Similar results were obtained with the HMWP (10–27% radiation bound). The maximum RAST-binding activity with both *A. suum* (21%) and HMWP (27%) was located within the first 8 mm of the gel, a position which corresponded to that occupied by the two glycoprotein bands described above (fig. 2b). In contrast, the RAST-binding activity of LMWP (10–15%) was evenly distributed along the gel. Allergenicity, determined by RAST analysis, of *A. suum* perienteric fluid fractionated on Sephacryl S-300 paralleled Pc activity (fig. 4a).

Allergenic Cross-Reactivity

RAST inhibition studies showed that extracts of *T. canis* and various life-cycle stages of *N. brasiliensis*

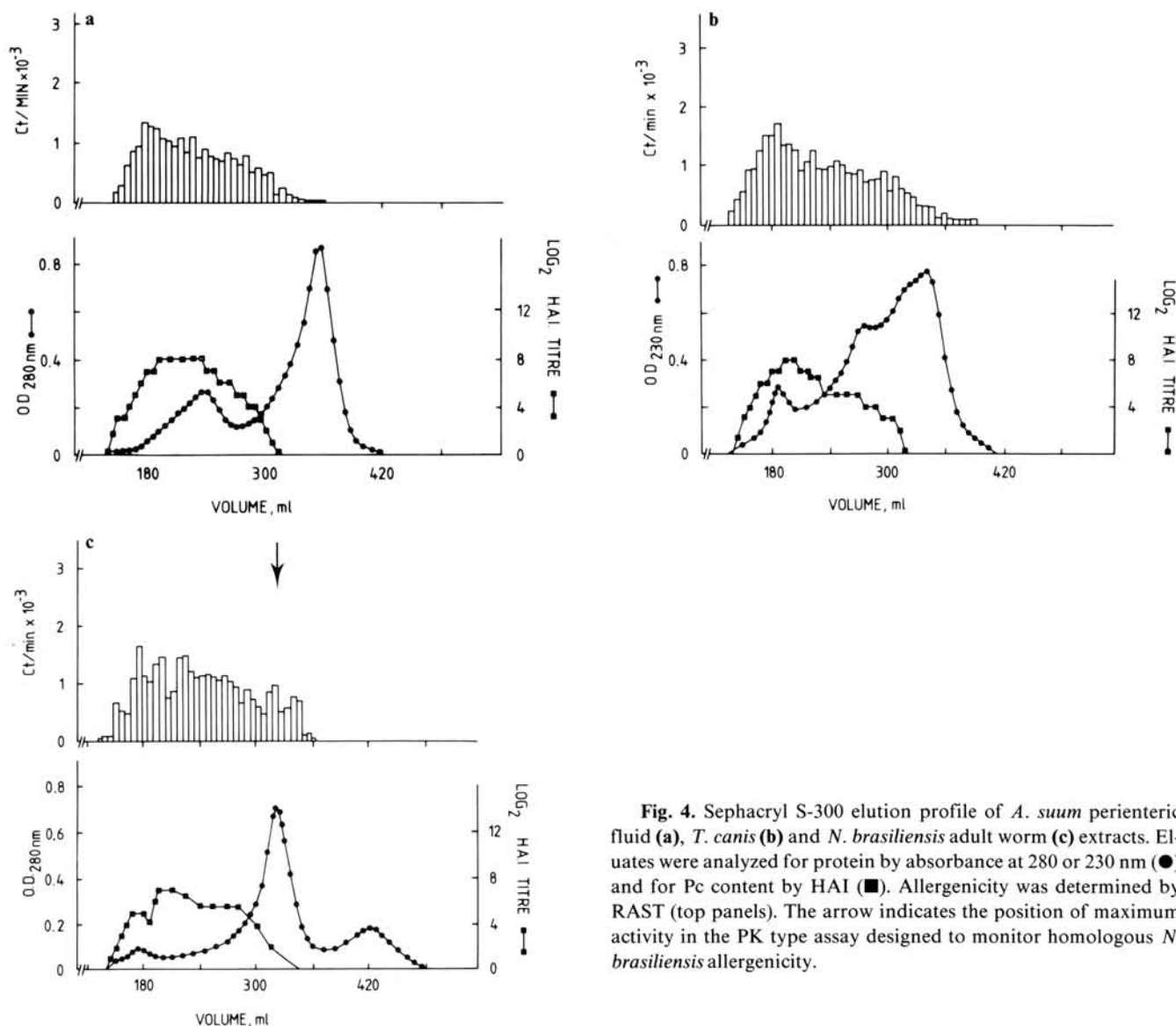


Fig. 4. Sephacryl S-300 elution profile of *A. suum* perienteric fluid (a), *T. canis* (b) and *N. brasiliensis* adult worm (c) extracts. Eluates were analyzed for protein by absorbance at 280 or 230 nm (●) and for Pc content by HAI (■). Allergenicity was determined by RAST (top panels). The arrow indicates the position of maximum activity in the PK type assay designed to monitor homologous *N. brasiliensis* allergenicity.

cross-reacted to varying degrees (although 9- to 500-fold less potent) with *A. suum* perienteric fluid (table II). In contrast, extracts of *S. ratti* and *S. matthei* were judged not to cross-react, with the exception of *S. matthei*, all the extracts examined contained relatively large amounts of Pc (table II).

Sephacryl S-300 gel filtration studies on extracts of *T. canis* and the available live-cycle stages of *N. brasiliensis* showed that the cross-reacting allergens identified by RAST binding were heterogeneous with regard to molecular weight (fig. 4b, c). The allergenicity profiles obtained were similar to that demonstrated

by *A. suum* (fig. 4a) in that the majority of the cross-reacting allergens were of high molecular weight. In addition, the Pc-profiles for *T. canis* and all stages of *N. brasiliensis* (fig. 4, only data for *N. brasiliensis* whole worm extract shown) were similar to that obtained with *A. suum* and paralleled allergenicity. Confirmation of this allergenic cross-reactivity was obtained by RAST-inhibition analyses of the various gel filtration fractions of *T. canis* and *N. brasiliensis* whole worm and larvae extracts. The most allergenic fractions corresponded, in general, with those containing the majority of Pc and were of high molecular

Table II. Allergenic cross-reactivity and Pc content of helminthic parasites

Parasite	RAST inhibition		
	amount required (in $\mu\text{g/ml}$) to give 50% inhibition of RAST ¹	relative potency ²	Pc content HAI units/mg protein
<i>A. suum</i>	152	100	315,985
<i>T. canis</i>	1,366	11.1	507,834
<i>N. brasiliensis</i>			
Adult worm	985	15.4	126,732
L ₃ larvae	3,588	4.2	81,541
Secretory/excretory	69,358	0.2	51,807
<i>S. ratti</i>	> 100,000	> 0.2	682,666
<i>S. mattheei</i>	> 100,000	> 0.2	124
Pc-BSA	> 100,000	> 0.2	146 $\times 10^9$

¹ Discs were coated with *A. suum* peritric fluid.

² Relative potency was expressed as a percentage of the *A. suum* peritric fluid.

weight (data not shown). The high molecular weight (approx. 600,000 daltons) fractions from the extracts examined were 20- to 50-fold more potent than the low molecular weight (approx. 20,000 daltons) fractions.

Homologous Allergenicity

The above studies indicated that the cross-reactivity between *A. suum* and the other helminths studied was primarily associated with the high molecular weight components. Studies were, therefore, performed to determine whether the molecular weight distribution of homologous allergens was similar to that observed for the cross-reacting allergens using sera from *N. brasiliensis*-infected rats and *N. brasiliensis* extracts. These studies showed that limited PK activity was associated with the high molecular weight material which corresponded with that containing Pc in all three *N. brasiliensis* extracts examined. However, the majority of the activity was associated with low molecular weight components (approx. 20,000 daltons) where PC content was minimal (fig. 4c, only data for *N. brasiliensis* whole worm extract shown).

Allergenicity of the Hapten Pc

Because of the observed parallelism between allergenicity and the presence of the PC, the allergenicity of the hapten was investigated by RAST inhibition using a synthetic Pc-BSA conjugate but was shown not to be allergenic per se (table II).

Discussion

The studies described here showed that allergens from *A. suum* recognized using *Ascaris* RAST-positive sera from PNG donors were heterogeneous with regard to both molecular weight and charge as judged by gel filtration and PAGE in accordance with data from O'Donnell and Mitchell [15]. The HMWP of *A. suum* isolated by Sephadex G-200 gel filtration was approximately 15-fold more potent than the original extract as judged by RAST-binding studies and of approximately equal potency when judged by RAST inhibition. In contrast, the LMWP was approximately 30- to 40-fold less potent than the original extract using both RAST and RAST inhibition.

CRIE studies showed that the HMWP of *A. suum* contained 10 allergens compared with 6 found in the LMWP. PAGE studies, in combination with RAST, suggested that the two high molecular weight carbohydrate-staining components which remained at the top (cathode) of the gel after completion of electrophoresis may be allergens. Further studies are presently under way to isolate these components and determine their allergenicity.

A. suum extracts have been shown to cross-react allergenically with the nematode worms *T. canis*, *Haemonchus contortus* [2, 15, 23], *N. brasiliensis* [this study] and *Necator americanus* [2, 23] but not with cestodes and trematodes [15, and this study]. However, our data showed that *A. suum* did not cross-react with

the nematode *S. ratti*. This failure to demonstrate cross-reactivity may be real or reflect the use of a different life stage of the *S. ratti* lacking cross-reacting antigens which might otherwise be found with the mature worm.

The physicochemical nature of this cross-reactivity has not been previously examined in detail but studies described here indicate that maximum cross-reactivity occurs in association with high molecular weight (approx. 600,000 daltons) components and, in addition, parallels Pc activity. The presence of Pc, however, does not necessarily mean that two parasite extracts may be allergenically related since while *S. ratti* contained high levels of Pc it did not cross-react with *A. suum*. Moreover, the hapten per se was not allergenic, an observation consistent with previous studies employing Pc-containing allergens from the mite *Dermatophagoides pteronyssinus* [22].

PK studies using sera from rats infected with *N. brasiliensis* showed that whilst the cross-reactivity between this parasite and *A. suum* was predominantly associated with the high molecular weight components, homologous allergenic activity was primarily associated with low molecular weight allergens. In *N. brasiliensis* larval extract, however, and to a much lesser extent adult worm extract, PK activity was found with high molecular weight components also. The presence of low molecular weight (approx. 20,000 daltons) *N. brasiliensis* allergens is consistent with previous studies in which a low molecular weight secretory/excretory antigen was isolated from *N. brasiliensis* adult worm with an apparent molecular weight of about 12,000 daltons [19]. However, the reasons underlying the failure of the high molecular weight components to trigger a more prominent IgE response in *N. brasiliensis*-infected rats are, at present, unknown.

While Pc is present in phospholipids and in phospholipid moieties of vertebrate lipoproteins, and in bacterial and fungal C-substances, the identity of the PC carrier in parasites was, until recently, unknown. Although some studies suggested that the parasite Pc carrier in *N. brasiliensis* was analogous to pneumococcal C-substance [18], recent studies from our laboratory [12] with both *N. brasiliensis* and *A. suum* indicate that lipoproteins may be the major carrier of Pc in helminthic parasites. These studies showed that low, high and very high density lipoproteins of both *A. suum* and *N. brasiliensis* with apparent molecular weights ranging from 470,000 to $>1 \times 10^6$ daltons

contained Pc, with the majority being present in the very high density lipoprotein fraction (470,000–610,000 daltons).

On the basis of the molecular weight distribution data, our unpublished observations that the high density, Pc-containing lipoproteins of *A. suum* are allergenic, and reactivity with the lectin Con A (which is known [11] to react with lipoproteins), it is likely that the major allergens in *A. suum* peritenteric fluid are lipoproteins and that Pc is present as the phospholipid phosphatidylcholine, a moiety known to be widely distributed amongst parasites [7].

Such conclusions are not without precedence since it has been demonstrated that both the trematode *Fasciola hepatica* and the cestode *Echinococcus granulosus* both contain at least two lipoprotein allergens [10,16]. The relationship between the apoproteins of nematode lipoproteins described here and the apoproteins from cestode and trematode lipoproteins previously described is not known but RAST inhibition data [this study; 15] would suggest that there will be little cross-reactivity. Interestingly, however, data obtained from cestode and trematode studies indicate that both species specificity and cross-reactivity may be defined on the basis of the lipoproteins contained therein [3,4]. These data would be consistent with the cross-reactivity data described here for the nematodes. The isolation and characterization of lipoproteins from *A. suum* is currently under way.

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