An Investigation of the Allergens of *Ascaris lumbricoides* using a Radioallergosorbent Test (RAST) and Sera of Naturally Infected Humans: Comparison with an Allergen for Mice Identified by a Passive Cutaneous Anaphylaxis Test

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Abstract

The identification of those components of *Ascaris lumbricoides* (var. *suum*) body fluid (ABF) which are IgE-inducing antigens (allergens) was found to depend on the type of assay used. By use of the radioallergosorbent test and sera from humans naturally infected with *A. lumbricoides*, it was found that ABF contains a range of allergens with a variety of isoelectric points and molecular weights. Some cross-reactions were demonstrated between the allergens of *A. lumbricoides* and *Toxocara canis*. On the other hand, when a passive cutaneous anaphylaxis assay was used with sera from mice sensitized by nasal inhalation of ABF plus *Bordetella pertussis* vaccine, it was found that only one relatively pure fraction of ABF was involved. This consisted of some of the largest protein molecules in ABF: it had a molecular weight of approximately 360 000 (subunits 140 000 and 220 000), an isoelectric region of 8.0-8.4, and was clearly very different from the allergens isolated from ABF by other workers.

ABF and its fractions need to be freshly prepared and to be kept in the presence of reducing agents to avoid rapid deterioration and irreproducibility of some results.

The relatively pure allergen fraction of ABF, isolated after identification by the passive cutaneous anaphylaxis assay and tested in mice, was found to be T cell-stimulating but was non-mitogenic. Access to the circulation after intranasal administration appeared to be highly restricted.

Introduction

There is no doubt that products of parasites are recognized by a host’s immune system and are responsible for its stimulation (Warren 1971; Cohen 1974, 1976; Dineen *et al.* 1974; Ogilvie and Love 1974; Ogilvie and Worms 1976; Wakelin and Lloyd 1976). In the rational development of immunodiagnostic reagents and vaccines, antigens responsible for the various aspects of antiparasite immune responses need to be identified, isolated, and individually studied for biological effects (see Soulsby 1975a, 1975b). One characteristic response to helminth infections in man and animals is an increase in the amount of specific and non-specific circulating IgE (Johansson *et al.* 1968; Orr and Blair 1969; Jarrett and Stewart 1972; see Dessaint *et al.* 1975a, 1975b for summary). The efficacy of specific IgE in helminth elimination from a host is controversial (see Ogilvie and Parrott 1977; Musoke *et al.* 1978). Rose (1973), referring to the results of Phills *et al.* (1972) of deliberate infection of man with *Ascaris*, suggested that 'the stimulation of IgE... (is)... most probably related to the mechanisms which are directed towards ridding the body of parasites generally'. However, Jacobson *et al.* (1977) have produced evidence that mice lacking antibody production potential are still able to eliminate the helminth *Nippostrongylus brasiliensis*. 
The functions of IgE need not be confined to immediate hypersensitivity. Capron et al. (1975) have evidence that specific IgE is involved in the binding of macrophages to the schistosomules of *Schistosoma mansoni*. They suggested that 'specific IgE anti-schistosome antibodies may have a central role in the immunity of the rat to this parasite, and are not merely concerned with the concurrent development of immediate-type hypersensitivity'.

In this paper we report the identification and characterization of some of the components of the helminth *Ascaris lumbricoides* (var. *suum*) (*Ascaris*) to which humans respond by production of IgE when infected naturally. Two assay systems were employed to identify these allergens—a radioimmunoassay for specific IgE, i.e. the radioallergosorbent test [RAST (Wide et al. 1967)], using sera of infected New Guineans (cf. Turner et al. 1975), and a heterologous passive cutaneous anaphylaxis (PCA) test in rats using sera from mice sensitized by nasal inhalation of *Ascaris* body fluid (ABF) plus *Bordetella pertussis* vaccine (BPV). Sensitization by injection (and, to a lesser extent, infection) has been used previously by several workers looking for IgE-inducing antigens (allergens) in *Ascaris* (Ambler et al. 1972, 1973a, 1973b; Hussain et al. 1972, 1973; Bradbury et al. 1974; Strejan 1975; Kuo and Yoo 1977).

Some immunobiological properties of an allergen isolated from ABF after identification by the PCA test were compared with those of human gammaglobulin, an antigen known not to be a strong allergen. In addition, the RAST system was used to study cross-reactions between the allergens of *Ascaris* and *Toxocara canis*, since such cross-reactions cause confusions in the immunodiagnosis of helminth infections (see Cohen 1974; Soulsby 1975a).

**Acronyms and Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ABF</td>
<td><em>Ascaris</em> body fluid</td>
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<tr>
<td>Allergen</td>
<td>IgE-inducing antigen</td>
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<tr>
<td><em>Ascaris</em></td>
<td><em>Ascaris lumbricoides</em> (var. <em>suum</em>)</td>
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<tr>
<td>BBS</td>
<td>Borate-buffered saline: 6·18 g H₃BO₃, 9·54 g Na₂B₄O₇·10H₂O and 29·2 g NaCl per litre; pH 8·2.</td>
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<td>BPV</td>
<td><em>Bordetella pertussis</em> vaccine</td>
</tr>
<tr>
<td>CNBr-Sepharose-ABF</td>
<td>CNBr-Sepharose to which ABF has been bound</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Da.SI</td>
<td>Fraction from ABF which is not retarded by DEAE-cellulose and then elutes at the exclusion volume of Sephadex G200.</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid (disodium salt)</td>
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<tr>
<td>HGG</td>
<td>Human gammaglobulin</td>
</tr>
<tr>
<td>N.B.</td>
<td>Serum from an infected New Guinean which gave the highest reading in the RAST test</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline: 0·15 M NaCl, 0·01 M phosphate (1·07 g Na₂HPO₄, 0·39 g NaH₂PO₄·2H₂O, 8·5 g NaCl per litre); pH 7·2.</td>
</tr>
<tr>
<td>PBS-reducing buffer</td>
<td>PBS + 10 mM mercaptoethanol, 0·5 mM DTT, 5 mM EDTA at pH 7·2</td>
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<tr>
<td>PCA</td>
<td>Passive cutaneous anaphylaxis</td>
</tr>
<tr>
<td>RAST</td>
<td>Radioallergosorbent test</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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Methods

Collection of ABF

*Ascaris* worms from freshly slaughtered pigs were washed in physiological saline (0·15 M NaCl) at room temperature. Their ends were cut off and their body fluid allowed to drain into a measuring cylinder containing 45 ml mercaptoethanol, 6·7 mg DTT, and 3·75 ml 0·1 M EDTA titrated to c. pH 7·4. 75 ml ABF was collected over c. 1 h. The solution was then centred in mercaptoethanol, 0·5 mM in DTT and 5 mM in EDTA. It was stored in a stoppered glass container at 4°C for no longer than 3–4 weeks and checked regularly by the nitroprusside test to ensure the continued presence of reducing agent in the medium. A precipitate which formed after a few days settled to the bottom of the container and was excluded from further consideration. ABF used in mice to produce the 'stock reaginic sera' (see below) was stored at −20°C.

Preparation of Extracts from Other Helmints

Extracts of *Fasciola hepatica*, *Toxocara canis*, *Macracanthorhynchus hirudinaceus* and *Haemonchus contortus* were made by grinding freshly collected worms with PBS-reducing buffer in a Potter-Elvehem tissue homogenizer.

Concentrations of Extracts and Fractions

Extracts and fractions were concentrated by ultrafiltration under nitrogen using UM2 or UM10 membranes in Diaflo cells (Amicon Corporation, Mass., U.S.A.) at 3 × 10⁵ Pa. When it was required to remove salt or change to a lower ionic strength (for example in the preparation of samples for isoelectric focusing), dialysis was carried out using 18/32 Visking cellulose dialysis tubing.

Fractionation of ABF Extracts

The fractionations of ABF were monitored by PCA tests, isoelectric focusing, SDS gradient or gradient acrylamide gel electrophoresis, and cellulose acetate electrophoresis.

(i) On DEAE-cellulose

Whatman microgranular cellulose (DE52—Reeve Angel and Co., Kent, England) was used in accordance with the manufacturer's directions. The freshly collected ABF (i.e. collected within 24 h of use) was given three 1-h dialyses at room temperature against 20 volumes of starting buffer (10 mM sodium borate, 10 mM mercaptoethanol, 0·5 mM DTT, 1 mM EDTA, pH 8·2) before loading on to the DE52 column.

(1) Analytical procedures. Details of this are given in the legend to Fig. 1.

(2) Preparative procedures. 75 ml freshly collected ABF was dialysed and fractionated at 25°C in a jacketed column (2·5 cm diameter by 25 cm; Pharmacia, Sweden). The column was washed with starting buffer, the protein solution not bound was made 0·15 M in NaCl, and was concentrated to 30 ml using a UM10 membrane. It was stored either in this solution or dialysed against PBS-reducing buffer at pH 7·2, and kept at 4°C. If further purification was to be carried out on the run-through peak, 15 ml was loaded on to a Sephadex G200 column in PBS-reducing buffer the same day that the worms were collected. The other 15 ml was loaded the next day.

(ii) By gel filtration

Fractionation of whole ABF on Sephadex G200 was used. The details are given in the legend to Fig. 2. For all the preparative work after initial fractionation on DE52, a column of Sephadex G200 in PBS-reducing buffer was used. The required fractions were pooled, concentrated to c. 1% (w/v) protein concentration, and stored at 4°C.

(iii) By ammonium sulphate gradient solubilization

The procedure used here followed very closely that developed by King (1972). 33 ml freshly collected ABF was diluted with 167 ml of PBS-reducing buffer at pH 7·2. 22 g Celite (Fisher Scientific Co., New Jersey, U.S.A.) was added and the solution made 80% saturated with respect to ammonium sulphate by the addition of 112 g of this salt. The mixture was then packed in a column (2·5 cm diameter) jacketed at 25°C. When required, bulked fractions were dialysed against PBS-reducing buffer, concentrated using a UM10 membrane to an absorbance at 276 nm (A₂76) of 7–10 and stored at 4°C.
(iv) By electrophoresis on cellulose acetate

(1) Analytical procedures. Separations of ABF or fractions from it were done using a Beckman Microzone apparatus. The protein solutions were dialysed against the electrophoresis buffer before loading on to the cellulose acetate membrane. Approximately 1–5 µg protein was loaded. The staining and washing procedures were those of Blagrove et al. (1975). The buffers used were: (a) pH 8·8, Chemtron No. 3 buffer (Chemtron, Italy) consisting of 10·3 g sodium veronal (the disodium salt of diethyl barbituric acid) and 1·34 g veronal; (b) pH 9·2, 0·05 M sodium phosphate; and (c) pH 10·1, 0·05 M β-alanine, 10 mM mercaptoethanol, 0·001 M EDTA and sodium hydroxide to the required pH.

(2) Preparative procedures. Preparative fractionations of ABF on cellulose acetate were done using Cellogel blocks (Chemtron, Italy) and their recommended No. 3 buffer at pH 8·8 (see previous paragraph). 250 µl dialysed ABF was loaded and run at room temperature for 18 h at 110 V and 10 mA. Prints were taken with strips of cellulose acetate and stained, as in the analytical runs, to find the positions of the protein bands. The block was then cut into strips and the protein solutions removed by centrifugation through Millipore filters. The fractions were dialysed against routine PBS-reducing buffer and concentrated by ultrafiltration.

High pH buffers were not used for preparative work because of the slow loss of biological activity (as determined by the PCA assay) at higher pH values.

(v) By isoelectric focusing

(1) Analytical procedures. Analytical isoelectric focusing was carried out on slabs of acrylamide gel on subbed plates (Ilford, England), 21 by 16 cm, according to the method of Awdeh et al. (1968) and Williamson (1973). The gels were made 2% (w/v) in Ampholines (LKB-Produkter, Sweden) and 1 mM in mercaptoethanol. The stock solutions were made according to Spencer and King (1971).

To get the acrylamide gels to stick well to the subbed plate, a Perspex former and a silicone rubber gasket (Robinson 1972) were used. The coloured markers normally used were chosen from 1% (w/v) solutions of bovine serum albumin plus bromphenol blue, horse myoglobin, sperm whale myoglobin and cytochrome c (Radola 1973). The separated protein bands were first made visible by soaking the focused plate in 10% (w/v) trichlороacetic and, if staining with dye was then required, the method of Vesterberg (1972, and as given in the LKB-Produkter Ampholine–polyacrylamide gel plate instructions) was superimposed on this.

(2) Preparative procedures. Preparative isoelectric focusing was done on a Multiphor apparatus (LKB-Produkter, Sweden). The manufacturer’s instructions were followed, supplemented by reference to the work of Radola (1974). Ultrodex (LKB-Produkter, Sweden) was used as the supporting medium and mercaptoethanol (10 mM) was added to the Ultrodex–Ampholine slurry. On 100-ml gels, ABF (0·25–5·00 ml), dialysed first against 1% (w/v) glycine at pH 7·2, was mixed in with the whole slurry. On occasions, 50-ml gels were used and six different samples were loaded by the microscope slide technique of Radola (1973). Cytochrome c (isoelectric point 9·3), loaded to a preparative gel by microscope slide at the anodic end, was often used as a marker to indicate when a run should be stopped. Paper prints were taken to find the positions of the focused protein bands. Small samples of the focused gel were mixed with water to measure pH values.

The protein fractions were eluted with two 3-ml lots of PBS-reducing buffer, and 100-µl samples were taken for amino acid analysis. Fractions were concentrated where necessary by ultrafiltration. Ampholines were removed when required by dialysis or by passage of the fractions through columns of Sephadex G25 or G75. When 5 ml of fresh ABF was focused on a 100-ml (initial volume) gel the eluted fractions had A₂₇₀ values in the range 0·5–1·0 and could be used without further processing for PCA testing.

Other Analytical Methods for Characterization of ABF Fractions

(i) Electrophoresis in SDS acrylamide gradient gel

Polyacrylamide gradient gels (Gradiapore, 2·5–25% concave gradient, variable cross-linkage) were obtained from Gradient Pty Ltd, Lane Cove, N.S.W. They were run at room temperature in their glass cells (Margolis and Kenrick 1968) in a single-cell Gradiapore apparatus. The gels were pre-run with 0·025 M phosphate (pH 7·2) containing 0·05% (w/v) SDS for 1 h before loading the samples (Weber and Osborn 1969). The gels were normally run at least until the bromophenol blue marker added to the solution reached the bottom of the cell.
For solutions containing 1% (w/v) of a particular protein, 20 μl was diluted to 100 μl with water and 20 μl of a solution comprising 0·25 m phosphate, pH 7·0, 5% (w/v) SDS, 5% (v/v) mercapto-ethanol was added. After heating in boiling water for 2 min, 40 μl of loading solution [20% (v/v) glycerol in 0·05 m phosphate buffer, pH 7, plus bromophenol blue] was added and 2–5 μl (c. 2–5 μg protein) was added per slot of Gradipore’s sample spacer. Samples to be examined were prepared from stock protein solutions each day. A run at 50 mA took approximately 5 h. After removal from the glass cell, the gel was stained for 1 h in a solution of 0·15% (w/v) Coomassie G250 in 50% (v/v) methanol and 7·5% (v/v) acetic acid and destained overnight in 5% (v/v) methanol–7·5% acetic acid with a piece of woollen blanket to absorb the dye. If the samples being examined contained Ampholines it was necessary to remove them by washing the gel with three or four changes, every 2–3 h, in 10% (w/v) trichloroacetic acid before staining with the dye solution.

(ii) Electrophoresis in 5% acrylamide gels containing SDS
These slab gels were run similarly to the gradient gels as described in the previous section.

(iii) Electrophoresis in 5% acrylamide gradient and non-gradient gels in buffer at pH 10·2
These were slab gels as above and were made at pH 7 in 0·025 m phosphate buffer. They were pre-run for 1 h in 50 mM β-alanine, 10 mM mercaptoethanol, 5 mM EDTA plus sodium hydroxide to pH 10·2, before loading the samples and were stained as described above.

Protein Contents of ABF and ABF Fractions
The protein contents of ABF and fractions from it were calculated from the amino acid content. It was assumed that half of the side-chain carboxyl groups of aspartic acid and glutamic acid were amidated, and that the nitrogen contents of the protein components of ABF were 16·6%. Elution profiles were followed by measuring absorbance at 276 nm. When solutions were too cloudy to measure absorbance they were diluted 1:9 with 98–100% (v/v) formic acid. This gave optically clear solutions.

Iodination of Isolated ABF Proteins
The methods employed for the labelling of the isolated ABF proteins with 125I were those of Greenwood et al. (1963) using chloramine T, and those of Bolton and Hunter (1973) using iodinated 3-(4-hydroxyphenyl)propionic acid N-hydroxsuccinimide ester as acylating reagent. Carrier-free 125I, at a concentration of 100 mCi/ml, was purchased from the Radiochemical Centre, Amersham, England. Human gammaglobulin (Commonwealth Serum Laboratories, Melbourne, Vic.) and concanavalin A (Con A) (A grade, Calbiochem, San Diego, California) were iodinated using the chloramine-T method. All antigens were iodinated to a level of not more than one 125I atom per molecule of protein and then diluted to provide a dose per mouse of 10–20 μg protein of approximately 105 cpm (< 0·1 μCi/μg). All labelled proteins were more than 80% precipitable with trichloroacetic acid and were used within 3–4 weeks of iodination.

Binding of ABF Extracts to CNBr–Sepharose
CNBr–Sepharose (Pharmacia, Sweden) was used in accordance with the manufacturer’s instructions. Fresh ABF was dialysed for 2 h using Visking cellulose dialysis tubing against changes of BBS, pH 8·2 (1 ml of the dialysed solution was made to 5·3 ml with BBS). The A276 was c. 5·1. After binding to 500 mg CNBr–Sepharose overnight, the A276 of the supernatant was 0·90, i.e. over 80% of the material (as measured by absorbance) had been bound. The CNBr–Sepharose–ABF complex was stored at 15 mg/ml at 4°C in buffer consisting of 0·2% (w/v) ovalbumin (made according to the method of Kekwick and Cannan 1936), 1% (v/v) Tween 20, 0·01% (w/v) sodium azide, 0·5 mM DTT, 10 mM mercaptoethanol, 5 mM EDTA in PBS. The reducing agents were removed before using the CNBr–Sepharose–ABF complex in RAST estimations.

RAST Determinations
Basically the methodology of Aronsson and Wide (1974) was used for the RAST allergen assay. Their ‘direct’ and ‘indirect’ methods were compared on separated fractions of ABF. Since no substantially different conclusions could be drawn from results using the two methods, only the indirect method is reported here.
(i) Standard curve

To obtain a standard curve, 50 µl of serum N.B. from an Ascaris-infected human (obtained by Dr P. Booth of Port Moresby during a routine collection of blood in an area of high Ascaris prevalence in New Guinea) was stood for 1 h at 37°C with 50 µl of ABF solutions dialysed against, and serially diluted with, PBS. 1 mg of CNBr-Sepharose–ABF complex, freshly washed with PBS, was added in 0.5 ml incubation buffer. The latter consisted of 0.2% (w/v) ovalbumin, 1% (v/v) Tween 20, and 0.1% (w/v) sodium azide in PBS. After reaction on a rotating wheel for 3 h at room temperature, the tubes were centrifuged, the CNBr-Sepharose–ABF complex washed three times with PBS and once with incubation buffer and left finally in approximately 0.5 ml incubation buffer. 50 µl of 125I-labelled Phadebas anti-IgE (Pharmacia, Sweden) was added and the tubes rotated overnight. The samples were then washed as indicated above and counted for 2 min on a Packard Autogamma counter with an efficiency of approximately 70%.

To obtain the standard curve, log10 ([C0 − Cx]/C2) was plotted as ordinate and log10 x as the abscissa, where C0 is the number of counts bound in the test tube without inhibition by the diluted ABF solutions minus unspecifically bound radioactivity (umbilical cord serum, kindly supplied by Dr J. Barrie, from the Royal Women’s Hospital, Melbourne, was substituted for the N.B. serum to get this value), Cx is the number of counts bound in the tube with the solutions containing various numbers of Ascaris units minus unspecifically bound radioactivity, and x is the number of Ascaris units in the diluted ABF solution added to a particular assay (cf. Aronsson and Wide 1974).

(ii) Allergic activity of ABF fractions

The activities of various ABF fractions were compared by calculating their inhibitory capacity in terms of Ascaris units from the standard curve. An Ascaris unit was defined as the inhibitory capacity of the solution obtained when fresh ABF was diluted 1000 times, i.e. 1 ml of diluted solution contained 1 µl of ABF.

Total IgE

The total IgE in some of the sera was determined using the Phadebas IgE test kit (Pharmacia, Sweden).

Stability of the Biologically Active Fraction Da.S1 as Assessed by the PCA Test

(i) Stability in absence of mercaptoethanol, DTT and EDTA

To remove mercaptoethanol, DTT and EDTA quickly from the stored biologically active fraction, Da.S1, 0.4 ml of the protein was passed through a column (0.87 cm diameter by 10 cm) of Sephadex G200 in PBS. The protein fraction (now free of reducing agent as judged by the nitroprusside test) was divided into four 1-ml aliquots and these were allowed to stand at room temperature. After periods of 0, 24, 48 and 88 h, one tube was made 10 mM in mercaptoethanol and 5 mM in EDTA to stop any reaction and stored at 4°C until the experiment was completed. The four samples together with the original material were then examined by SDS acrylamide gradient gel electrophoresis, and also tested for residual PCA-eliciting activity.

(ii) Stability to enzymes

1% (w/v) solutions of the Da.S1 material were treated for 3 h with enzyme (1% w/w). The Da.S1 material (Fig. 3) after treatment by trypsin, chymotrypsin, or pronase was examined as above.

(iii) Stability to high pH

Fraction Da.S1 was stood at room temperature for periods of up to 24 h at pH 10.2 in 50 mM β-alanine–NaOH, 10 mM mercaptoethanol, 5 mM EDTA, 0.5 mM DTT. Samples removed were brought to pH 7.2 by dialysis against PBS and examined as above for residual PCA-eliciting activity and for the appearance of bands in the SDS acrylamide gradient gel electrophoresis pattern.

Biological Testing of ABF Protein Fractions

(ii) Animals and intranasal administrations

Unless otherwise stated, 7–10-week-old CBA/H, BALB/c, (CBA/H × BALB/c)F1 and hypothyemic (nude) BALB/c–nu/nu and CBA/H–nu/nu female mice were used throughout. Their derivation from a specific pathogen-free facility and subsequent conventional maintenance have
been described in detail (Mitchell et al. 1976; Mitchell 1977). Rats used were 100–200-day-old inbred Lewis rats, bred and maintained in a conventional colony (Mitchell 1976). Antigens and BPV (Commonwealth Serum Laboratories, Melbourne) were administered intranasally in a volume of 50 μl normal saline or mouse tonicity PBS containing $1 \times 10^9$–$2 \times 10^9$ Bordetella pertussis organisms. Mice were anaesthetized with a 2:1 (v/v) mixture of ether:chloroform and the antigen plus BPV applied to the nose (Mitchell 1976). At various time intervals after intranasal administration of labelled antigens, a sample of blood was taken from the orbital plexus, or organs were removed, and counted for 10 s in a gamma counter. The administered dose was also counted at each time interval and the data expressed as the percentage of administered radioactivity estimated to be present in the blood stream or in each organ.

(ii) Stock reaginic sera

For identification of active ABF fractions in a rat PCA assay, two stock pools of sera have been used. Batch 1 was a pool of serum from 12-week-old (CBA × BALB/c)F₁ female mice each given 1 mg ABF plus $10^9$ BPV organisms intranasally and bled 35 days later. Batch 2 was a pool of serum from BALB/c-μ/μ female mice given a pool of mesenteric lymph node and thymus cells from female BALB/c mice (one donor per recipient) intraperitoneally and 500 μg ABF plus $2 \times 10^9$ BPV organisms intranasally. These mice were bled 16 days later, when the titre of circulating heat-labile reagins (IgE) is known to be high (Mitchell 1976).*

(iii) PCA tests using ABF fractions and also stock reaginic sera

ABF fractions (at $A_{276}$ of 1-0) were tested in individual rats which had been injected 18–24 h previously with 50 μl 1:5 batch 2 serum in saline (titre $> 1:125$) and/or 50 μl neat batch 1 serum (titre $= 1:25$). These serum amounts were chosen to increase the sensitivity of the assay and to ensure that weakly active ABF fractions would be detected. 1 ml ABF and fractions, together with Evans blue in PBS, was injected intravenously and the skin reactions assessed at 30–45 min (Mitchell 1976).

(iv) Assay for delayed-type hypersensitivity

The ear assay for delayed-type hypersensitivity in mice described by Vadas et al. (1975) was used, the basis of the read-out being the increased accumulation of radioactivity in the left ear of sensitized mice injected with antigen relative to the right ear which is un.injected or injected with an irrelevant antigen. Data are expressed as the ratio of radioactivity in the left ear to that in the right ear, a ratio of more than 1·6 being unequivocally positive.

(v) Microculture mitogenicity assay

The diluents, preparation of viable cell suspensions, and mitogens used in this assay have been described in detail by Shortman et al. (1977). Cells were set up in microtitre trays, incubated at 37°C in a humidified CO$_2$ incubator, and 0·1 μCi $^{125}$I-labelled 5-iodo-2-deoxyuridine (Radiochemical Centre, Amersham, England) was added per well on day 2 and harvested using an Otto Hiller harvester (Madison, Wisconsin, U.S.A.) on day 3. The data are expressed as the stimulation index using the means of triplicate cultures (see second footnote to Table 4).

Results

Stability of Ascaris Allergens

ABF and its fractions in PBS were found to be unstable with time either frozen or maintained at 4°C. This instability was shown by the disappearance of bands and

* Several consistent differences were noted between these two sera even though the reaginic activities detected in the 24-h rat PCA assay were totally heat-labile. The titre of batch 2 serum was higher than that of batch 1 ($> 1:125$, cf. 1:25), particularly when 1 mg of an active fraction of ABF plus 5–10 mg Evans blue in saline was used to elicit the PCA reaction. Of some interest was the observation that the PCA reaction, using a fixed dilution of serum, was positive in the case of batch 1 serum at 2 min after antigen and Evans blue injection to the rats, and reached maximum intensity at approximately 15 min. In the case of the higher titred batch 2 serum, reactions were first noted after an interval of 10 min and reached maximum intensity (which was greater than with batch 1 serum reactions) after approximately 25 min. Presumably this difference reflects a higher average combining site affinity, but lower amounts of IgE antibodies, in the day 35 serum from intact mice (batch 1) compared with day 16 serum from partially reconstituted nude mice (batch 2 serum).
the development of smeariness in the patterns obtained by SDS gradient gel electrophoresis, gradient gel electrophoresis, and isoelectric focusing on acrylamide gel. Gradient gel patterns in the absence of SDS showed the formation of aggregates. So, in the present work, fresh ABF was obtained every 2–3 weeks, and it was found necessary to keep it and its fractions in the presence of reducing agent—10 mM mercaptoethanol and 0·5 mM DTT were used and 5 mM EDTA was also present. Under these conditions, the RAST profiles of separated fractions of ABF were found to be reproducible. Solutions of ABF and purified fractions were stored at 4°C and, even in the presence of the reducing agent, showed a deterioration over periods longer than about 2 months. This requirement of reducing agent for stability of an allergen was also noted by Thompson (1972) when isolating mast cell degranulating substance, and Willadsen and Williams (1976) found that it was necessary to use a reducing agent when studying an allergen from the cattle tick.

Precipitation occurred in salt-free solutions of ABF or the fraction Da.S1. It is for this reason that isoelectric focusing was not the ideal method of separation for ABF proteins since the technique requires the absence of salt. Certainly fraction Da.S1 could be seen to be precipitated when isoelectrically focused in acrylamide gels and, at times, it did so over quite a wide pH range, as shown in the upper right hand photographs of Figs 1–3. When fraction Da.S1 was eluted from a preparative isoelectric focusing run it was found to be impossible to get sharp bands on a re-run; only vague protracted smears were obtained. This points to the occurrence of some irreversible behaviour of Da.S1 in the absence of salt at its isoelectric point. Irreproducible behaviour was also found when fraction c corresponding to Da.S1 was recovered after fractionation of ABF by ammonium sulphate gradient solubilization (Fig. 4).

Radioimmunoassays

(i) Serum IgE content

This was determined on various sera using the Phadebas IgE test kit (Table 1). Sera from each of four monkeys infected with embryonated Ascaris eggs and from one injected with ABF plus B. pertussis as adjuvant gave values indistinguishable from zero using this test. However, the monkeys did have anti-Ascaris IgE in their sera as shown by PCA tests on other monkeys (see Hogarth-Scott and Feery 1976). We conclude that monkey IgE does not cross-react in the RAST test with Phadebas anti-human IgE. On the other hand, Ishizaka et al. (1969), using a precipitin test, did find evidence for cross-reaction between monkey and human IgEs.

By determination of the total IgE in N.B. serum before and after absorption with CNBr-Sepharose–ABF it was found that the ABF-specific IgE was less than 4% of the total IgE (cf. Dessaint et al. 1975a, 1975b).

(ii) Standardization of the RAST test for IgE-binding allergens

The RAST standard curve plotted according to Aronsson and Wide (1974) is shown in Fig. 5. Standard curves from older ABF were parallel to, and to the left of, this curve. Using the serum from N.B. and 50 000 cpm 125I-labelled RAST anti-IgE (from the Phadebas kit), some 6000 cpm were taken up by 1 mg of CNBr-Sepharose–ABF. Approximately 93% of these counts were abolished by prior incubation of the N.B. serum with ABF. Umbilical cord serum obtained in Melbourne took up about 5% of the counts that N.B. serum did, and this value was not decreased
Allergens of *Ascaris lumbricoides*

Fig. 1. Lower: Gradient elution of ABF (3 ml) from a column of Whatman DE52 DEAE-cellulose (15 cm by 0.9 cm diameter) at 25°C. Fraction size 7.2 ml, 12-min fractions. Linear gradient with 100 ml on either side of the gradient device. Starting buffer was 0.01 M borate, 0.03 M NaCl, 10 mM mercaptoethanol, 0.5 mM DTT, 1 mM EDTA at pH 8.5, and the limit buffer contained an additional 0.37 M NaCl. The gradient was applied after tube 8. Histograms are the RAST activity of individual tubes. Upper: Acrylamide gradient gel electrophoresis patterns at pH 10.2 of ABF and of fraction a + b and fraction c + d from the DEAE-cellulose fractionation (left); SDS acrylamide gradient gel electrophoresis patterns of ABF and fractions a, b, c, and d (centre); isoelectric focusing patterns, pH 3.5–10.0, made visible according to the method of Vesterberg (1972), of ABF and fractions a, b, c, and d (right). I, iron-containing bands.
Fig. 2. Lower: Fraction of ABF (8 ml, undialysed) on a column of Sephadex G200 (135 cm by 2·3 cm diameter) in PBS-reducing buffer. Fraction size 9·4 ml, 30-min fractions. Blue dextran came out at 244 ml and peak 1 at 271 ml. The carbohydrate contents of the fractions were determined by the anthrone method on 1-ml aliquots of combined tubes as indicated. Histograms are the RAST activity of various tubes. Upper: SDS gradient gel electrophoresis patterns (left) and isoelectric focusing patterns, pH range 3·5-10·0 (right) of ABF and fractions 1, 3 and 4. I, iron-containing bands. At the extreme upper left is shown the pattern for proteins used as markers for molecular weight. My, myosin (molecular weight 220 000); B, bovine plasma albumin (67 000), whose dimer (B2) could normally be seen; O, ovalbumin (45 000); C, carbonate dehydratase (30 000); Mg, myoglobin (17 000).
by prior absorption of the cord serum with ABF. Pooled sera of people allergic to grass pollen and with high IgE content (1250 units IgE/ml) bound only some 12% as many counts as did N.B. serum, and this fell to 5% when the serum was pre-reacted with ABF. It is possible that one (or more) of the sera contributing to this high IgE serum pool was derived from an *Ascaris*-infected individual (or from an individual infected with some cross-reacting helminth such as *Toxocara canis*).

We were completely dependent on the quality of the Phadebas 125I-labelled anti-IgE used in our work. When we labelled commercially available anti-IgEs ourselves,
the RAST results were very different in that the RAST curve of elution profiles of ABF did not follow the protein curve and could not be interpreted in a simple manner. This was probably due to the fact that we were unable to purify our preparation by immunoadsorption as does the manufacturer of the Phadebas preparation.

Allergens in ABF as determined by RAST and Specific IgEs in Sera of Humans Naturally Infected with Ascaris

It can be seen from the various methods of separation applied to ABF [i.e. fractionation by DEAE-cellulose (Fig. 1), Sephadex gel filtration (Fig. 2), a combination of both (Fig. 3), ammonium sulphate gradient solubilization (Fig. 4), isoelectric focusing (Fig. 6), and cellulose acetate electrophoresis (Fig. 7)] that there are many

![Graph and Table](https://example.com/graph.png)

**Fig. 4. Lower:** Fractionation of ABF (33 ml) at 25°C on a column (2.5 cm diameter) by ammonium sulphate gradient elution. Fraction size 9.1 ml, 20-min fractions. Linear gradient (applied at tube 20) with 405 g buffer on either side of gradient device. Starting buffer was PBS-reducing buffer 80% saturated with respect to ammonium sulphate. Limit buffer was PBS-reducing buffer. 500 μl of each fraction was diluted with 1 ml PBS before measurement of absorbance. 1 ml of the combined tubes as indicated was tested for carbohydrate content, the minus signs indicating 10 μg/ml. ABF had a value of 14.7 mg/ml with glucose as standard. Histograms are RAST activity of various tubes. **Upper:** SDS gradient gel electrophoresis patterns of ABF and of fractions a–d obtained from it by the ammonium sulphate fractionation.
proteins in ABF differing in size and isoelectric point which have their own specific IgEs in the sera of a human naturally infected with *Ascaris*. Only that group of small molecules in the third peak off Sephadex G200 (Fig. 2) were exempt (as a group). The serum used to monitor this separation was N.B. A similar picture emerged when sera from two different naturally infected individuals (N.B. and N.C.) were compared using separation on Sephadex G200, and also when these were compared with sera from J.A.W., a sensitized laboratory worker unlikely to have been infected with

### Table 1. IgE content of various sera, and relative RAST activities against ABF bound to CNBr–Sepharose

<table>
<thead>
<tr>
<th>Serum</th>
<th>IgE (i.u./ml)</th>
<th>Relative RAST activity$^A$ (cpm)</th>
<th>Serum</th>
<th>IgE (i.u./ml)</th>
<th>Relative RAST activity$^A$ (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.A.W.</td>
<td>2875</td>
<td>10 880</td>
<td>N.G.$^E$</td>
<td>2000</td>
<td>2820</td>
</tr>
<tr>
<td>Pari</td>
<td>1900</td>
<td>4230</td>
<td>W.A.$^F$</td>
<td>1175</td>
<td>1380</td>
</tr>
<tr>
<td>Minj$^b$</td>
<td>295</td>
<td>1800</td>
<td>00074$^G$</td>
<td>$&gt;$ 5000</td>
<td>4050</td>
</tr>
<tr>
<td>N.A.$^E$</td>
<td>270</td>
<td>1200</td>
<td>D.R.$^H$</td>
<td>620</td>
<td></td>
</tr>
<tr>
<td>N.B.$^E$</td>
<td>4150</td>
<td>5900</td>
<td>Monkey injected with ABF$^I$</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>N.C.$^E$</td>
<td>700</td>
<td>5610</td>
<td>Monkeys 1–4 infected with <em>Ascaris</em>$^J$</td>
<td>$&lt;$ 20</td>
<td></td>
</tr>
<tr>
<td>N.D.$^E$</td>
<td>425</td>
<td>1800</td>
<td>Cord blood (pooled)$^K$</td>
<td>40</td>
<td>300</td>
</tr>
<tr>
<td>N.E.$^E$</td>
<td>3400</td>
<td>5070</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^A$ These values were all reduced to c. 300–400 cpm if the samples of serum were incubated first with ABF.

$^B$ Provided by Dr R. S. Hogarth-Scott, I. C. I. Research Laboratories, Melbourne, from a laboratory worker sensitized to *Ascaris*.

$^C$ Bulked sera from Pari village in Papua New Guinea, provided by Dr E. Dennis, University of Papua New Guinea, Port Moresby.

$^D$ Serum from Minj village in Papua New Guinea, provided by Dr C. C. Curtain, Division of Chemical Technology, CSIRO, Melbourne.

$^E$ Individual sera from inhabitants of an *Ascaris*-infected area of New Britain, provided by Dr P. Booth, Red Cross Blood Transfusion Service, Port Moresby.

$^F$ Bulked sera from persons with allergy to grass pollen, provided by Dr K. J. Turner, St Margaret’s Hospital, Perth.

$^G$ Serum from Singapore, provided by Dr R. S. Hogarth-Scott.

$^H$ From a person with visceral larva migrans and infected with *Toxocara canis*, provided by Dr R. S. Hogarth-Scott.

$^I$, $^J$ Provided by Dr R. S. Hogarth-Scott.

$^K$ Provided by Dr J. Barry, Pathology Unit, Royal Women’s Hospital, Melbourne.

*Ascaris* (R. S. Hogarth Scott, personal communication). It is difficult to attribute the fact that there are many IgE-inducing antigens identified in ABF in natural infections to ‘potentiation’ which has been shown to occur with non-worm antigens when a worm infection follows, since this type of potentiation is thought to depend on a live worm infection (Orr and Blair 1969; Jarrett and Stewart 1972; Smith *et al.* 1973; see also review by Tada 1976). However, there could conceivably be potentiators in ABF which are effective even if administered through the nose or skin. Such must be the route by which J.A.W. was sensitized.

* A recent paper (Tsuji *et al.* 1977) reported IgE antibodies to *Ascaris* antigens in a person experimentally sensitized with *Ascaris suum* antigens. The RAST test was used.
Cross-reactions between Extracts of Ascaris lumbricoides and Toxocara canis and Other Helminths

To investigate cross-reacting antigens in helminths using RAST, extracts of the various worms were reacted with N.B. or J.A.W. serum to see if this had any effect on the number of counts of $^{125}$I-labelled anti-IgE eventually taken up by the CNBr-Sepharose-ABF. It was found that extracts of the cestode Taenia saginata, the acanthocephalon Macracanthorhynchus hirudinaceus, and the trematode Fasciola hepatica had no effect, but extracts of the nematodes Toxocara canis (50 µl of extract of $A_{270} = 9.6$ was used in the test) and Haemonchus contortus (50 µl of an extract of $A_{270} = 16.2$ was used) reduced counts normally given by serum N.B. by approximately 62 and 60 % respectively. This indicated a considerable degree of cross-reaction. It was of interest that there was no cross-reaction given by T. canis that had been stored in a deep-freeze for 1–2 years before extraction. The extract used in the present work was from freshly collected worms.

Another way of investigating cross-reacting antigens in helminths is to use sera from humans with certified helminth infections in place of the N.B. serum (Table 1) from an Ascaris-infected human which we used in our routine RAST tests. Sera were therefore obtained from nine humans with certified helminth infections as indicated in Table 2. Although these sera were from humans from Ascaris-free areas, it is not certain that any one of them may not have had a helminth infection which did cross-react with specific anti-Ascaris IgE, as well as the helminth under consideration. Hence, a negative cross-reaction is a definite result, but a positive cross-reaction cannot be interpreted with certainty with regard to the specific helminth being surveyed. From Table 2 it seems that the correct conclusion to be drawn is that any IgE in sera from humans infected with the trematode Fasciola hepatica or the cestodes Echinococcus granulosus and Taenia saginata did not react with Ascaris allergens. The sera with the positive values means that these persons had a cross-reacting co-existing helminth infection as well as the one listed in Table 2. The positive result for cross-reaction with the serum from a person infected with Toxocara canis is evidence for the sharing of allergens between Ascaris and T. canis (Sadun 1972, 1976; Herzig 1974; de Savingy 1975; Hogarth-Scott and Feery 1976). Material
in both peaks 1 and 2 (Fig. 2) from the Sephadex G200 fractionation of ABF reacted in the RAST test using serum from *T. canis*-infected D.R. This suggests that there are several cross-reacting allergens in *Ascaris* and *T. canis*.

**Table 2. Cross-reactions of helminths as determined by RAST**

ABF bound to CNBr-Sepharose was incubated with sera from nine humans infected with various helminths before reaction with 125I-labelled anti-IgE in the standard RAST test. Values expressed are percentages of those given when serum N.B. was used in the standard RAST test (see Table 1). Individual values for each infection are given

<table>
<thead>
<tr>
<th>Serum from human infected with:</th>
<th>Relative RAST activity (%)</th>
<th>Serum from human infected with:</th>
<th>Relative RAST activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fasciola hepatica</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td><em>Taenia saginata</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td><em>Echinococcus granulosus</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Toxocara canis</em>&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sera from these five persons provided by Dr J. R. L. Forsyth, Department of Microbiology, University of Melbourne.

<sup>b</sup> Sera from these three persons provided by Dr M. Rickard, Department of Paraclinical Sciences, Veterinary Clinical Centre, University of Melbourne.

<sup>c</sup> Provided by Dr R. S. Hogarth-Scott, I.C.I. Research Laboratories, Melbourne. This is the D.R. serum of Table 1.

**Allergens in ABF as Determined by Heterologous Mouse–Rat PCA tests**

From the various methods of separation applied to ABF and depicted in Figs 1–4, 6 and 7 it can be seen that there was only one compound in ABF (although we have some evidence for isoallergens) to which mouse IgE was directed when the mice were sensitized by nasal inhalation of ABF and BPV. It was termed Da.S1 or the PCA allergen. The yield of Da.S1 was determined by amino acid analysis. Some 52% of ABF (c. 21 mg/ml) was not retarded by DEAE-cellulose (peak Da. Fig. 1).

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>PCA activity</th>
<th>Presence of bands of Da.S1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RAST activity A.U.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>A.U./mg protein</th>
<th>Iron colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++++</td>
<td>+++++</td>
<td>32</td>
<td>23</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>+</td>
<td>41</td>
<td>22</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>12</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>—</td>
<td>1·2</td>
<td>1</td>
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<tr>
<td>5</td>
<td>—</td>
<td>—</td>
<td>1·4</td>
<td>1</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

<sup>a</sup> cf. the two characteristic bands observed in SDS gradient gel electrophoresis (see Fig. 3, fraction 1).

<sup>b</sup> *Ascaris* units.

Fig. 7. Pattern of electrophoresis of ABF after an analytical run on cellulose acetate at pH 8·8. Arrow indicates loading point. The PCA activities and RAST values (see tabulation) are taken from a preparative run (1 ml ABF) in which five fractions were collected, each in a volume of 1·5 ml. These corresponded closely to those marked on the figure, although it seems as if there is some overlapping of fraction 1 into fraction 2 in the preparative run. When Da.S1 was run on cellulose acetate it showed only the slowest moving band (fraction 1).
Fig. 6. Isoelectric focusing pattern, pH range 3·5–10·0, of a preparative run (100-ml bed of Ultrodex) with 5 ml ABF for 16 h at 250 V and 10°C and then for 2 h at 1000 V. Fractions were bulked as shown in the tabulation below into fractions a–e and after dialysis and concentration to 3·7 ml, RAST activity (on a 1:1 dilution), absorbance at 276 nm, and protein concentrations were determined and PCA tests carried out on these bulked fractions. Results obtained were as follows (n.d., not determined):

<table>
<thead>
<tr>
<th>Fraction No.:</th>
<th>15</th>
<th>14</th>
<th>13</th>
<th>12</th>
<th>11</th>
<th>10</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>6</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH:</td>
<td>4·28</td>
<td>4·57</td>
<td>4·94</td>
<td>5·31</td>
<td>5·62</td>
<td>5·99</td>
<td>6·39</td>
<td>6·75</td>
<td>7·19</td>
<td>7·56</td>
<td>7·88</td>
<td>8·23</td>
<td>8·54</td>
<td>8·95</td>
<td>9·23</td>
</tr>
<tr>
<td>Combined fraction</td>
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<td></td>
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<td></td>
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<tr>
<td>$A_{276}$</td>
<td>1·04</td>
<td>1·90</td>
<td>1·44</td>
<td></td>
<td>0·58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0·36</td>
<td></td>
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<tr>
<td>Protein (mg/ml)</td>
<td>0·71</td>
<td>2·44</td>
<td>1·47</td>
<td>0·62</td>
<td>0·20</td>
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<tr>
<td>RAST activity</td>
<td></td>
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<td></td>
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<tr>
<td><em>Ascaris</em> units</td>
<td>20</td>
<td>78</td>
<td>96</td>
<td></td>
<td>26</td>
<td>5·2</td>
<td></td>
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</tr>
<tr>
<td><em>Ascaris</em> units/mg protein</td>
<td>28</td>
<td>32</td>
<td>65</td>
<td></td>
<td>42</td>
<td>26</td>
<td></td>
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<tr>
<td>PCA activity</td>
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<tr>
<td>Da.S1 bands$^A$</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

$^A$ i.e. presence in SDS gradient gel electrophoresis of bands characteristic of Da.S1.
Of this, some 18% was excluded by Sephadex G200 (peak Da.S1, Fig. 3), giving an overall yield of Da.S1 from ABF of 9·4%. The yield calculated using $A_{276}$ was approximately the same, and extinction coefficients, $E_{1%}^{1%}$, of ABF and various fractions were found to be approximately 10–11, in agreement with the work of Kuo and Yoo (1977).

**Some Biochemical Properties of Da.S1**

(i) **Molecular weight and subunit structure**

Da.S1 consisted of a single peak when examined by sedimentation in an ultracentrifuge and had an $S_{20,w}^0$ value of c. 10·6 in PBS (Fig. 8). When the molecular weight was determined by the meniscus depletion analysis of sedimentation equilibrium, it was found to be 300 000–400 000 plus some small amount of material of high molecular weight. This was consistent with the SDS gradient gel pattern (Fig. 3, upper centre, fraction 1) where the compound Da.S1 gave two characteristic bands of molecular weight approximately 160 000 and 220 000, apparently subunits of a fraction of molecular weight 380 000. Whereas Da.S1 sometimes consisted of two or three adjacent bands on a gradient gel pattern (Figs 1 and 3, upper left) and two or three bands on polyacrylamide disc gel electrophoresis (Fig. 9), it was found that the same two bands were obtained from all these various fractions when they were reduced and examined by SDS gradient gel electrophoresis (see Figs 1 and 3, upper centre; Fig. 9). Therefore the original bands probably represent very similar proteins. Since the two characteristic bands seen in the SDS gradient gel of Da.S1 correlated with the PCA activity of ABF through several methods of separation used, it is unlikely that the fraction responsible for PCA activity is an impurity attached to a large amount of another protein.
(ii) Stability

Da.S1 was unstable in the absence of reducing agents, even at 4°C, and its progressive degradation in their absence is shown in Fig. 10. This progressive change in its characteristic SDS gradient gel pattern with time was paralleled by a loss in PCA-eliciting activity. Gradient gel patterns in the absence of SDS showed the formation of aggregates.

Treatment with pronase or chymotrypsin gave a similar but much accelerated picture. Trypsin had a somewhat slower effect than these two enzymes in that the characteristic bands and PCA activity had disappeared in less than 24 h. So far we have been unable to isolate a small 'active fragment' from Da.S1 such as has been done with codfish allergen M (Elsayed et al. 1972). King and co-workers (see King 1976 for summary) have not found it possible yet to get an active fragment from allergen E from ragweed.

At pH 10·2 and room temperature, Da.S1 again lost its SDS gradient gel pattern and PCA activity within 24 h. Under acid conditions the Da.S1 fraction precipitated. Also the PCA activity of Da.S1 was completely lost when it was labelled with \(^{125}\)I by the chloramine-T method of Greenwood et al. (1963). Thus for studies on the fate of labelled Da.S1 in vivo (see below) it was necessary to use the Bolton and Hunter (1973) reagent for iodination as this did not lead to loss of PCA-eliciting activity.

(iii) Amino acid composition

The predominant amino acids in the hydrolysate of Da.S1 were lysine, leucine, and aspartic and glutamic acids (Table 3). In this respect it was similar to other IgE-inducing antigens isolated from Ascaris by Ambler et al. (1973) and Hussain et al. (1973), as well as the mast cell degranulating substance isolated by Thompson (1972). There were also pronounced differences between the three allergen preparations as shown in Table 3.

The amino acid analysis of Da.S1 bears no resemblance to that of the ryegrass allergens, e.g. allergen E of King and co-workers (see King 1976), and Ra5 of Goodfriend and co-workers (see Roebber et al. 1975). Nor is there any resemblance to that of the cod fish allergen of Elsayed and co-workers (see Elsayed et al. 1972). In any case King (1976) and Marsh (1975) have dismissed the idea of there being any obvious similarity or distinguishing chemical features between allergens.

(iv) Carbohydrate content

Da.S1 had a very small carbohydrate content, as judged by the anthrone test on the fraction containing it obtained by ammonium sulphate gradient solubilization (Fig. 4, fraction c).

Some Immunobiological Properties of Da.S1

(i) Induction of reaginic response

In the experiments reported above, the active fraction of ABF, Da.S1, has been identified by elicitation of PCA reactions using pooled sera from mice sensitized to ABF plus BPV. The ability of Da.S1 to induce reagins to ABF in mice has also been examined. In two separate experiments we have observed an interesting dose effect related very closely to that reported by Strejan et al. (1973). CBA/H female mice were dosed intranasally with various amounts of Da.S1 or ABF, together with BPV,
and bled over a period of 30 days thereafter. High doses of ABF (i.e. 100–200 µg) were consistently and markedly superior to comparable doses of Da.S1 at inducing anti-ABF reagins. By contrast, 10–40 µg Da.S1 was superior to 10–40 µg ABF even though titres in both cases were low. Conceivably, 'potentiators' in ABF are responsible for promoting high-titred reagins and these potentiators are not present in the Da.S1 preparation. With an unstable allergen such as Da.S1 the potentiators in ABF may not have any immunological effects but may simply stabilize the allergen during in vitro storage or in vivo. ABF does not appear to have any influence on the fate of 125I-labelled Da.S1 plus BPV administered intranasally (see below).

Table 3. Amino acid analysis of ABF and of a purified biologically active fraction, Da.S1, from it

The fraction was separated on DEAE-cellulose (Fig. 1) and Sephadex G200 (Fig. 3). The values are given as residues of amino acid per 100 residues. All hydrolysates were carried out with constant-boiling HCl in vacuo for 24 h in the presence of mercaptoethanol to minimize the destruction of tyrosine and tryptophan (Matsubara and Sasaki 1969). No corrections have been applied for the destruction of serine and threonine or for the possible incomplete release of valine and isoleucine. n.d., not determined

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>ABF¹</th>
<th>Da.S1¹</th>
<th>Allergen²</th>
<th>Allergen³</th>
<th>Mast cell degranulating substance²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>9·6²</td>
<td>8·2²</td>
<td>17·4</td>
<td>12·0</td>
<td>14·2</td>
</tr>
<tr>
<td>Histidine</td>
<td>4·7</td>
<td>3·7</td>
<td>5·3</td>
<td>4·2</td>
<td>2·1</td>
</tr>
<tr>
<td>Arginine</td>
<td>3·3</td>
<td>5·9</td>
<td>1·3</td>
<td>2·1</td>
<td>3·3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9·3</td>
<td>10·9</td>
<td>7·2</td>
<td>11·3</td>
<td>7·2</td>
</tr>
<tr>
<td>Threonine</td>
<td>5·2</td>
<td>5·3</td>
<td>4·4</td>
<td>4·9</td>
<td>4·7</td>
</tr>
<tr>
<td>Serine</td>
<td>5·2</td>
<td>6·5</td>
<td>4·9</td>
<td>4·9</td>
<td>2·9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>16·5</td>
<td>10·8</td>
<td>17·4</td>
<td>12·7</td>
<td>15·9</td>
</tr>
<tr>
<td>Proline</td>
<td>3·6</td>
<td>4·0</td>
<td>2·2</td>
<td>4·2</td>
<td>6·2</td>
</tr>
<tr>
<td>Glycine</td>
<td>6·5</td>
<td>6·6</td>
<td>5·9</td>
<td>7·7</td>
<td>4·2</td>
</tr>
<tr>
<td>Alanine</td>
<td>9·1</td>
<td>7·4</td>
<td>8·5</td>
<td>10·6</td>
<td>8·7</td>
</tr>
<tr>
<td>Valine</td>
<td>5·3</td>
<td>6·6</td>
<td>3·8</td>
<td>6·3</td>
<td>7·0</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>n.d.</td>
<td>1·6²</td>
<td>1·8</td>
<td>0</td>
<td>2·8</td>
</tr>
<tr>
<td>Methionine</td>
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<td>1·2²</td>
<td>1·0</td>
<td>0</td>
<td>1·3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4·3</td>
<td>5·0</td>
<td>3·3</td>
<td>4·2</td>
<td>4·2</td>
</tr>
<tr>
<td>Leucine</td>
<td>9·9</td>
<td>8·9</td>
<td>9·9</td>
<td>9·9</td>
<td>8·2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3·2</td>
<td>3·0</td>
<td>3·1</td>
<td>2·1</td>
<td>2·9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3·3</td>
<td>4·2</td>
<td>1·7</td>
<td>2·8</td>
<td>4·0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
<td>1·4</td>
<td>0</td>
<td>0·3</td>
</tr>
</tbody>
</table>

* Average of duplicate hydrolysates on each of two separate preparations.
* Values of Ambler et al. (1973) included for comparison.
* Values of Hussain et al. (1973) included for comparison.
* A pure compound isolated by Thompson (1972) from Ascaris. The values have been recalculated for comparison with the present results.
* There was a peak immediately after lysine and about one-third of the area of lysine.
* Determined as cysteic acid and methionine sulfone in separate analyses by the method of Moore (1963).
* The area of the glucosamine peak of the amino acid trace was about 1/30 that of lysine.

(ii) Cell-mediated immnunity

After intranasal administration of ABF plus BPV, T cell-injected nude mice responded by exaggerated production of circulating IgE relative to intact mice
Nude mice themselves are absolute non-responders and it is assumed from this difference between nude and T cell-injected nude mice that ABF activates T cells. The capacity of Da.S1 to induce a delayed-type hypersensitivity reaction in mice was determined using the ear assay as follows. Two groups each of five male and five female BALB/c mice were given a subcutaneous injection of cyclophosphamide on day 0, then on day 2 each group received an injection into the footpads of either Freund’s complete adjuvant or adjuvant plus 100 µg Da.S1. 5-Fluorodeoxyuridine and 125I-labelled 5-iodo-2-deoxyuridine were injected intraperitoneally and 20 µg Da.S1 was injected into the left ear of all mice in each group on day 7. The left–right ear ratio of radioactivity, read on day 8, was as follows (values expressed as arithmetic means ±s.e.): 1·03±0·03 and 1·00±0·09 for male and female mice respectively given adjuvant alone and 2·60±0·22 and 3·58±0·44 for male and female mice respectively given adjuvant plus allergen. These results clearly indicate that Da.S1 is a ‘strong’ T cell-stimulating antigen in mice, at least when injected together with Freund’s complete adjuvant.

Table 4. Failure to detect mitogenic activity of biologically active fraction Da.S1 in a microculture assay

<table>
<thead>
<tr>
<th>Substance added (amount per well)</th>
<th>Responding spleen cell suspensionA</th>
<th>Mean stimulation indexa</th>
<th>Expt 1</th>
<th>Expt 2c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A (0·5 µg)</td>
<td>CBA/H</td>
<td>47</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA/H–nu/nu</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> lipopolysaccharide (10 µg)</td>
<td>CBA/H</td>
<td>14</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA/H–nu/nu</td>
<td>24</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Phytohaemagglutinin-P (10 µl, 1:80)</td>
<td>CBA/H</td>
<td>15</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA/H–nu/nu</td>
<td>&lt;1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Da.S1 (3–400 µg, expt 1; 0·4–200 µg, expt 2)</td>
<td>CBA/H</td>
<td>&lt;2·2</td>
<td>&lt;1·1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA/H–nu/nu</td>
<td>&lt;1·7</td>
<td>&lt;1·3</td>
<td></td>
</tr>
</tbody>
</table>

A 1·5 × 10⁵ or 3·0 × 10⁵ viable nucleated cells per well.
B Ratio of the radioactivity incorporated into the cells in the presence and absence of known mitogens or the test material.
C In this experiment 40 µg Da.S1 did not significantly inhibit the concanavalin A responsiveness of CBA/H spleen cells nor the E. coli lipopolysaccharide responsiveness of CBA/H–nu/nu spleen cells.

(iii) B and T cell mitogenicity

Recently we have found that Con A is an excellent allergen when administered intranasally or injected subcutaneously to certain strains of mice (Mitchell and Clarke, unpublished data; see also Gollapudi and Kind 1975). Since Con A is a strong T cell mitogen, it was of interest to determine whether Da.S1 was also mitogenic. Using a microculture assay based on that described by Shortman et al. (1977), in which Con A leads to stimulation indices in excess of 20, Da.S1 was found not to be a T cell mitogen (Table 4). In addition, experiments with Escherichia coli lipopolysaccharide and spleen cells of nu/nu mice indicated that Da.S1 was also not a B cell mitogen.

(iv) Fate after intranasal administration

Conceivably, potent airborne allergens may be molecules which have affinity for components of the mucous membranes or other tissues in, or associated with, the
respiratory tract. We have tested whether Da.S1 given intranasally (i.e. simulating airborne exposure) together with BPV is retained to a greater extent than molecules such as HGG, a weak allergen which does not lead to high-titred early or sustained reagin responses after administration by this route. Immediately after intranasal administration of $^{125}$I-labelled Da.S1 and HGG to anaesthetized mice more than 70% of the radioactivity was present in the lungs and increasing amounts were later found passing through the stomach, presumably as a result of ingestion via the trachea, of antigen expelled from the lungs. Radioactivity measured in the blood stream of mice given HGG intranasally is not derived from the gastrointestinal tract since less than 0·2% of administered radioactivity was present 24 and 48 h after intranasal administration of $^{125}$I-labelled Da.S1 and HGG (Mitchell and O'Donnell, unpublished observation).

![Fig. 11. Percentages of administered radioactivity estimated to be in the blood, and calculated from a 100–200-µl blood sample taken from the orbital plexus, at various time intervals after intranasal administration of $2 \times 10^9$ BPV organisms plus 10–20 µg $^{125}$I-labelled Da.S1 (×) or $^{125}$I-labelled HGG (○) in CBA/H (- - -) and BALB/c female mice (——). Additional data have been included at 48 h. There were three to seven mice per point, and the geometric mean and s.e. of the mean are indicated. $^{125}$I-labelled Con A was also studied and the curve was similar to that of $^{125}$I-labelled Da.S1.](image-url)
The BPV effect, which is not T cell-dependent, since it occurs in nude mice given $^{125}$I-labelled Da.Sl, presumably reflects increased access of antigen into the grossly inflamed lung of BPV-injected mice (Lehrer et al. 1976). Although it is unwise to overemphasize these observations made in short-term experiments in which the label has not been proven to be still associated with the administered antigen, the data suggest that absorption of Da.Sl from the lungs is the opposite to what we have anticipated, i.e. it has limited access, and that blood-borne dissemination is especially limited. It remains to be determined whether this is a fundamental property of potent airborne allergens such as ABF and Con A or environmental grass pollens, the property being one which confines allergenic molecules, in small quantities, to respiratory tract tissues. We conclude from this limited study of the immunobiological properties of Da.Sl that this allergen is a T cell-stimulating, non-mitogenic molecule which does not appear to have a high penetrability in the lungs after intranasal administration.

Table 5. Retention of radioactivity in the lungs of female mice 48 h after intranasal administration of $^{125}$I-labelled HGG or $^{125}$I-labelled Da.Sl, with or without BPV

10–20 µg labelled antigen was administered. Data are expressed as geometric means. The upper and lower limits of the standard error are obtained by multiplying and dividing by the number in parentheses.

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Number of mice</th>
<th>Labelled antigen</th>
<th>Radioactivity in lungs (as % of administered radioactivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ BPV</td>
</tr>
<tr>
<td>CBA/H</td>
<td>4</td>
<td>HGG</td>
<td>12.9 (1.2)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>HGG</td>
<td>10.1 (1.2)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>HGG</td>
<td>12.5 (1.1)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4</td>
<td>HGG</td>
<td>44.1 (1.1)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>HGG</td>
<td>66.7 (1.2)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>HGG</td>
<td>21.4 (1.1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>HGG</td>
<td>16.1 (1.1)</td>
</tr>
<tr>
<td>CBA/H</td>
<td>4</td>
<td>Da.Sl</td>
<td>2.5 (1.4)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Da.Sl</td>
<td>3.5 (1.2)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Da.Sl</td>
<td>1.6 (1.2)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4</td>
<td>Da.Sl</td>
<td>9.6 (1.1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Da.Sl</td>
<td>12.6 (1.1)$^\text{A}$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Da.Sl</td>
<td>8.6 (1.1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Da.Sl</td>
<td>3.5 (1.2)</td>
</tr>
</tbody>
</table>

$^\text{A}$ This BPV effect was noted also in BALB/c–nu/nu mice in this experiment.

Discussion

The major finding in this work was that in a human naturally infected with *Ascaris* there were specific IgE antibodies, identified by RAST, to many *Ascaris* molecules of widely varying charge and size. In this respect the results are similar to those of Harris (1973, 1975), who isolated allergens from the trematode *Schistosoma mansoni*, and identified them by an active cutaneous anaphylaxis test, the Prausnitz–Kustner (P–K) test. This contrasts with the finding, using the PCA technique, that a single (or small number of closely related) compound(s) was responsible for the induction
of IgE in mice, an unnatural host for *Ascaris*, sensitized by nasal inhalation of ABF, an unnatural manner of sensitization to living helminths. The isolation of allergens from ABF, the 'universal allergen' (Rose 1973; Marsh 1975), has been the object of many workers (e.g. Ambler et al. 1974; Bradbury et al. 1974; Herzig 1974; Kuo and Yoo 1977). In most studies these allergens have been initially identified by PCA tests in rats with sera from rats that have been sensitized by injection of ABF with *B. pertussis* as adjuvant. Concerning the allergenicity of molecules during *Ascaris* infections, it was shown by Bradbury et al. (1974) that the allergen Asc-1 (Hussain et al. 1972, 1973), isolated from adult *Ascaris suum*, was 'present in all stages of the parasitic life cycle and was directly involved in stimulating reagin production during the migratory phase of the infection'. A few of our results, using embryonated *Ascaris* eggs which had been disrupted in a French pressure cell, showed similar RAST profiles, after Sephadex fractionation, to those obtained with ABF. Similar remarks apply to the material released by *Ascaris* worms when kept alive for a few days in saline. *Ascaris* allergens identified to date using the PCA test appear to have isoelectric points in the region 5–6 and molecular weights in the range 14 000–50 000 (Herzig 1974; see Kuo and Yoo 1977 for summary). However, in much of the work it was observed that there are other allergens in *Ascaris*, and the present work adds yet another *Ascaris* allergen (denoted Da.S1) to the list. With its isoelectric point in the range 8.2–8.4, a molecular weight of about 360 000, and its susceptibility to destruction by enzymes (cf. Ambler et al. 1974), Da.S1 is certainly different from the allergens isolated by other workers. It could not be converted to a PCA-eliciting molecule of molecular weight approximately 14 000 by enzyme treatment. The obvious conclusion from this paper and from the literature, in which multiple allergens have been recognized in other systems such as beech pollen (Ceska et al. 1972) and cow dandruff (Ceska and Hulten 1972), is that there is no unique chemical structure of allergens (King 1976; Tada 1976). If B^c cells are derived from B^c cells (Manning et al. 1976) there will certainly not be unique B^c cell-stimulating allergenic determinants.

It is being accepted (Marsh 1975; King 1976) that an allergen shows no characteristic differences from an antigen in properties such as isoelectric point and amino acid sequence (see also Aas 1978). The reviews by those two authors suggest that if a compound is to be an allergen then 40 000–60 000 is the upper limit of molecular weight because of the upper limit of mucosal membrane permeability. The finding in the present work of Da.S1 as a strong allergen with a molecular weight of approximately 380 000 (subunits 160 000 and 220 000) removes even this criterion of the characteristics of an allergen.

One of the principal objectives in the studies reported here was to isolate an allergen, free from non-allergenic molecules, with which to study several immunobiological properties of allergens. Obviously it is pointless to commence such studies with a mixture of antigenic molecules. The availability of the purified allergen Da.S1 enabled us to examine the fate of a labelled allergen after intranasal instillation, to examine the effects of added BPV on this fate, to examine the T cell-stimulating activities of the allergen, and to determine whether the allergen is a T cell mitogen. The latter point arose out of studies which have demonstrated that the T cell mitogen, Con A, is a potent allergen in some strains of mice but only when injected subcutaneously or given intranasally (G.E. Mitchell and A. E. Clarke, unpublished data). Results of various repeated experiments were clear cut: Da.S1 is not a mitogen, it is capable of inducing a strong delayed-type hypersensitivity response, and, most
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importantly, it appears not to be absorbed readily after intranasal administration. Most of the labelled Da.Sl appears to be expelled from the lungs (and swallowed) rather than absorbed into lung tissues and the blood stream. Retention in lungs of $^{125}$I-labelled HGG, a weak allergen, was greater than $^{125}$I-labelled Da.Sl over a 48-h (Table 5) or 5-7-day period (Mitchell and O'Donnell, unpublished observations). BPV increased the amount of $^{125}$I-labelled Da.Sl retained in the lungs but not the amount of $^{125}$I-labelled HGG. The conclusion seems warranted that the component of ABF which is most effective in inducing an IgE response in mice after intranasal administration is absorbed only in small amounts from lung airways.

All available evidence points to the fact that a more profitable study than the isolation of allergens in ABF may be the isolation of 'potentiators' of reagin production in ABF and other potent allergenic mixtures of molecules. Although Da.Sl is the only ABF allergen we can detect when mice are dosed intranasally with ABF plus BPV, Da.Sl is certainly inferior to ABF in high doses at inducing high-titred, persistent reagin responses in mice. Notwithstanding the importance of potentiators and adjuvants, there is one likely property of allergenic molecules which we wish to emphasize, this emphasis stemming from the highly restricted systemic dissemination of Da.Sl and Con A after intranasal administration and the allergenic potency of Con A injected subcutaneously. This is the property of affinity for tissue components at the major portals of entry of antigens—skin and mucous membranes. Restriction of T cell-dependent immune induction to these sites, rather than involvement of organized lymphoid structures such as the spleen, may well facilitate IgE production. Potentiators may be molecules or manipulations which increase access and retention of allergens to submucosal or subcutaneous sites (G. F. Mitchell and A. E. Clarke, unpublished data).

The present work suggests that for antigens of *Ascaris* to be stable they must be freshly prepared and kept in the presence of reducing agent. The ABF bound to CNBr-Sepharose was found to be stable for at least 3-4 months at 4°C in incubation buffer containing mercaptotoethanol and EDTA. However, ABF at 4°C in the presence of mercaptotoethanol and EDTA slowly lost RAST activity. If these conditions give stable antigens from other helminths, then the detection of IgE responses in parasitized individuals could be readily achieved using RAST. We wish therefore to point out the potential of this technique for the simple immunodiagnosis of helminth infection, just as it has become an accepted technique for the diagnosis of causative agents for atopic allergies (see Ceska and Hulten 1972; Aronsson and Wide 1974; Eriksson et al. 1976). Indications are that it would be better than immunodiagnostic tests based on IgG since preliminary work has shown that most sera from 16 people of Melbourne (an *Ascaris*-free area) when reacted with CNBr–Sepharose–ABF subsequently caused the uptake of a substantial number of counts when reacted with $^{125}$I-labelled anti-IgG. None of these sera gave such pseudo ‘*Ascaris*-positive’ results using RAST and Phadebas $^{125}$I-labelled anti-IgE.

With regard to the isolation and study of helminth antigens responsible for induction of host-protective immunities and eventual development of vaccines, a much more comprehensive range of methods must be employed for antigen identification and antibody determination than those used here. Promising lines of work appear, among others, from the results of Rickard and Adolph (1977) and Kwa and Liew (1977) (see also Gemmell 1976) who showed that metabolic excretions or secretions (ES antigens) from the cestodes *Taenia ovis* and *T. taeniaeformis* produced
immunity in hosts, and from the results of Silverman et al. (1962) and Rothwell and Love (1974) who showed that guinea pigs could be immunized against the nematode *Trichostrongylus colubriformis* in a similar manner. We are encouraged by such results to pursue the course of isolation of worm antigens involved in host protection.

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**References**


Allergens of *Ascaris lumbricoides*


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