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Surface labelling of *Strongyloides ratti*: stage-specificity and cross-reactivity with *S. stercoralis*

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SUMMARY

Various methods of radioiodination were compared for their efficacy in labelling the surface of *Strongyloides ratti* infective larvae and adult worms. The Iodogen method was chosen as the optimal technique for this parasite. The surface location of ^{125}-iodine was confirmed with light microscope autoradiography of transverse sections of labelled worms. Stage-specific surface components were identified when the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of infective larvae and adult worms were compared. Labelled surface molecules were solubilized with either the non-ionic detergent Triton-X-100, the anionic detergents sodium deoxycholate (DOC) or sodium dodecyl sulphate (SDS), or the cationic detergent cetyl trimethyl-ammonium bromide (CTAB). The CTAB extract yielded most labelled proteins that retained their antigenicity in an immunoprecipitation assay with hyperimmune mouse sera. Immunoprecipitation analysis with stage-specific mouse sera revealed that the surface of infective larvae is immunogenic and that there are no cross-reactions with adult worms. Adult worms resident in the intestine were not found to be immunogenic and showed a complete absence of reactivity. Antigenic determinants shared between *S. ratti* and *S. stercoralis* were identified. Patients infected with *S. stercoralis* precipitated bands with molecular weights 32 and 34 kD which were not reactive with normal sera. These reactions suggest the potential usefulness of the surface of *S. ratti* as a source of diagnostic antigens.

Keywords radiolabelling surface antigens *Strongyloides* stage-specificity cross-reactivity

INTRODUCTION

The roles of the surface structures of the nematode parasite, *Strongyloides ratti*, in the generation of protective immunity or the stimulation of antibodies useful for sero-diagnosis are uncertain. Infective larvae migrate through the tissues while adult worms reside in the mucosa of the small intestine. Little is known of the contribution of each of these stages to the development of immunity and the nature of those molecules responsible for such immunity has yet to be identified.

The surface of parasitic nematodes has been shown to be antigenic in many infected hosts (Philipp & Rumjaneck, 1984). Surface labelling techniques provide an opportunity to identify and characterize the surface proteins, to determine their antigenicity, and to isolate those which are relevant for the diagnosis of infection or the generation of protective immunity.

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Conventional techniques result in profiles of proteins visualized by SDS-PAGE. These patterns, however, depend not only on the parasite but also upon the labelling technique used and the method of solubilization employed to recover the labelled molecules. For these reasons, a range of labelling procedures and a variety of detergents were compared for their efficiency in labelling and retrieving surface antigens. In addition to using standard methods of solubilization with non-ionic (Triton-X-100) or anionic detergents (DOC and SDS), we also employed the cationic detergent, CTAB (cetrimide). This follows the observation of Pritchard *et al.* (1985) who showed recently that this agent was capable of selectively stripping surface antigens from a number of parasitic nematodes. Furthermore, these authors noted that the material released by CTAB retained its antigenicity as indicated by immunoprecipitation with immune sera.

Once the optimal labelling technique had been identified, experiments in mice infected with *S. ratti* were undertaken to examine the roles of infective larvae and adult worms in eliciting antibodies which would react with the surface molecules of either of those two stages in the life-cycle. Further, human sera

from normal individuals and patients infected with *S. stercoralis* were reacted with surface extracts of *S. ratti* infective larvae and adult worms to determine the level of cross-reactivity between the two species and to identify any diagnostically-useful antigens present in *S. ratti* preparations.

MATERIALS AND METHODS

Parasites and animals

A homogonic strain of *Strongyloides ratti* was maintained in female Sprague–Dawley rats supplied by the Animal Resource Centre, Murdoch, Western Australia as described previously (Dawkins *et al.*, 1980). Infective larvae were obtained from rat faecal cultures while adult worms were taken from the intestines of rats 7 days after infection (Grove & Northern, 1987). Sera were raised in female C57Bl/6J mice, 18–20 g in weight.

Immune sera

Hyperimmune sera for immunoprecipitation were prepared in mice exposed to either a complete infection, the systemic larval phase of infection, or the adult worm intestinal phase of infection, using a multiple infection schedule as described in Northern & Grove (1988). Human sera were obtained from patients with strongyloidiasis and normal sera were collected from uninfected individuals. Serum antibodies to *Strongyloides* were measured by ELISA as described elsewhere (Carroll, Karthigasu & Grove, 1981).

Iodination

Five iodination techniques were utilized. In order to permit comparison among these techniques and between the stages of the worms, 200 μCi of ^{125}I were used to label either 10 000 viable infective larvae or 500 viable adult worms; these quantities were based on the calculated relative surface areas of infective larvae and adult worms.

Chloramine T. Two-hundred microcuries of carrier-free radioactive iodine (^{125}I, Amersham, UK) plus 10 μl of a 1 mg/ml solution of chloramine T were added to 10 000 infective larvae or 500 adult worms. The mixture was allowed to react at room temperature for 2 min following which 10 μl of a saturated tyrosine solution were added to stop the reaction. The parasites were washed by centrifugation in Tris-HCl four times to remove any unbound iodine and then counted for radioactivity and viability (Hunter & Greenwood, 1962).

Lactoperoxidase. Two-hundred microcuries ^{125}I plus 7 μg lactoperoxidase (Sigma, St. Louis, MO) and 7 μg glucose oxidase (Sigma) were added to 10 000 infective larvae or 500 adult worms suspended in 200 μl Tris-HCl containing 20 mM D-glucose. The reaction was allowed to proceed at room temperature for 10 min and was terminated; the worms were washed and counted as before (Hubbard & Cohn, 1975).

Iodogen. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Pierce Chemical Co., Rockford, IL) was dissolved in methylene chloride to give a final concentration of 1 mg/ml then 100 μl aliquots were dispensed into 3 ml glass tubes and the solvent evaporated under a stream of nitrogen. Before use, the tubes were rinsed with Tris-HCl then 10 000 infective larvae or 500 adult worms in 200 μl Tris-HCl were added to each tube along with 200 μCi ^{125}I. The reaction continued for 10 min at room temperature with occasional agitation and was stopped and counted as described before (Fraker & Speck, 1978).

Bolton and Hunter reagent. The Bolton and Hunter reagent (*N*-succinimydil 3-4-hydroxy 5-^{125}I-iodophenyl propionate, Amersham) was used to label the parasites according to the manufacturer's instructions with some modification. Briefly, 200 μCi aliquots of the reagent were dispensed into 3 ml glass tubes and the solvent evaporated under nitrogen. A suspension of either 10 000 infective larvae or 500 adult worms in 200 μl 0·1 M borate buffer was added to the tube and incubated on ice for 10 min. The iodination reaction was stopped by the addition of 200 μl of 0·2 M glycine then the worms were washed in borate buffer as before (Bolton & Hunter, 1973).

Iodosulphanilic acid. Iodosulphanilic acid (New England Nuclear, Dupont Co., Boston, MA) was converted to the diazonium salt according to the manufacturer's instructions. Two-hundred microcuries ^{125}-iodosulphanilic acid in 100 μl phosphate buffer (PB) pH 7·5, was added to a suspension of 10 000 infective larvae or 500 adult worms in 100 μl PB. The mixture was incubated on ice for 15 min then the reaction was terminated by the addition of 10 μl of a saturated tyrosine solution. The worms were washed four times by centrifugation in PB then resuspended in Tris-HCl and counted for radioactivity and viability as before (Marshall & Howells, 1985).

Autoradiography of labelled, sectioned worms

Autoradiography was performed on 1 μm sections by the stripping film technique (Bogoroch, 1972) using Ilford K2 nuclear emulsion. The emulsion was exposed at 4°C and developed according to the manufacturer's instructions.

Solubilization of labelled proteins

After labelling, viable parasites were washed in fresh Tris-HCl, pH 7·4, then resuspended in 200 μl of either antigen preparation buffer (APB) (20 mM Tris-HCl pH 8·0, 5 mM ethylenediamine tetra-acetic acid, 2 mM phenylmethylsulphonyl fluoride and 100 units/ml aprotinin, Bayer Pharmaceuticals, Botany, Australia), 1% Triton-X-100 in APB, 1% DOC in APB, 1% SDS in APB or 0·25% CTAB in APB. They were placed in a 37°C incubator for 1 h and then the pellet of whole worms and supernatant fluid containing surface extracts were separated by centrifugation at 10 000 *g* for 5 min and the percentage of radioactivity in the soluble fraction was determined.

Immunoprecipitation of labelled peptides

The detergent extracts of labelled protein were preabsorbed with an equal volume of 10% protein-A Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Between 10^5–10^6 ct/min of the preabsorbed extract was added to 10 μl of each test serum in 100 μl NETT (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0·05% Triton-X-100, pH 7·4). These were incubated at room temperature for 2 h then 100 μl of 10% protein-A Sepharose CL-4B beads were added to each tube and incubation mixtures were rocked gently at room temperature for 30 min. Protein-A Sepharose beads with bound immune complexes were washed three times in NETT (Goding, 1983).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Samples for analysis by SDS-PAGE were solubilized for analysis by boiling for 5 min in SDS-PAGE sample buffer then subjected to SDS-PAGE as described by Northern & Grove (1987). Bio-rad pre-stained molecular weight standards were used (Bio-rad, CA) and ^{14}-C-labelled molecular weight stan-

Table 1. Efficacy of radiolabelling and viability of infective larvae and adult worms using various iodinating techniques

Label	Infective larvae ct/min (% viable)	Adult worms ct/min (% viable)
Chloramine T	$2{\cdot}4 \times 10^6$ (98)	$0{\cdot}14 \times 10^6$ (100)
Lactoperoxidase	$1{\cdot}2 \times 10^6$ (96)	$0{\cdot}014 \times 10^6$ (100)
Iodogen	$4{\cdot}0 \times 10^6$ (98)	$4{\cdot}0 \times 10^6$ (100)
Bolton and Hunter reagent	$0{\cdot}3 \times 10^6$ (0)	$3{\cdot}7 \times 10^6$ (0)
Iodosulphanilic acid	$0{\cdot}095 \times 10^6$ (100)	$0{\cdot}068 \times 10^6$ (100)

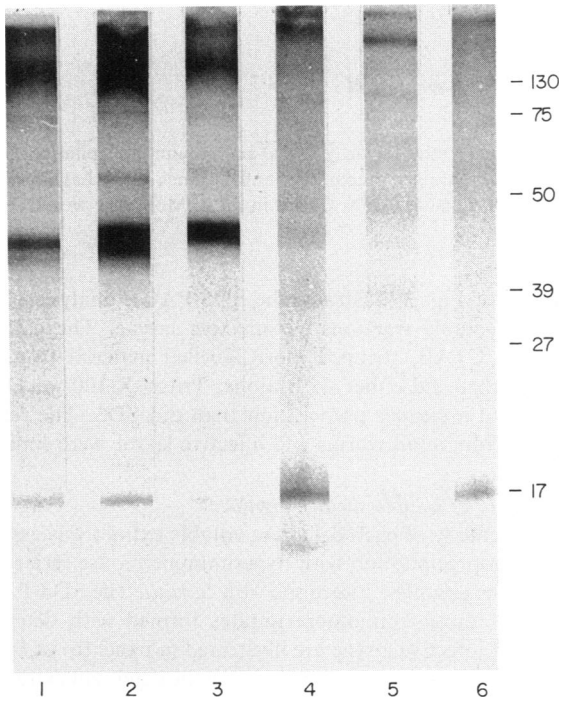

Fig. 1. SDS-PAGE analysis of infective larvae (lanes 1–3) and adult worms (lanes 4–6) by Iodogen (lanes 1 & 4), choloramine T (lanes 2 & 5) and lactoperoxidase (lanes 3 & 6). Molecular weight standards are indicated on the right (kD).

dards (Amersham) were used for molecular weight estimation on autoradiographs of SDS-PAGE. Protein bands were stained with Coomassie brilliant blue. The stained gels were dried and autoradiographed at −70°C using Dupont intensifying screens and X-Omat AR film (Eastman, Kodak, Rochester, NY).

RESULTS

Iodination of infective larvae and adult worms

Infective larvae and adult worms labelled by the five methods were counted for incorporated radioactivity and their viability assessed after the labelling procedure. These results are summarized in Table 1. The Iodogen labelling technique yielded the greatest amount of ^{125}I incorporated onto the surface of both infective larvae and adult worms and caused no loss of viability.

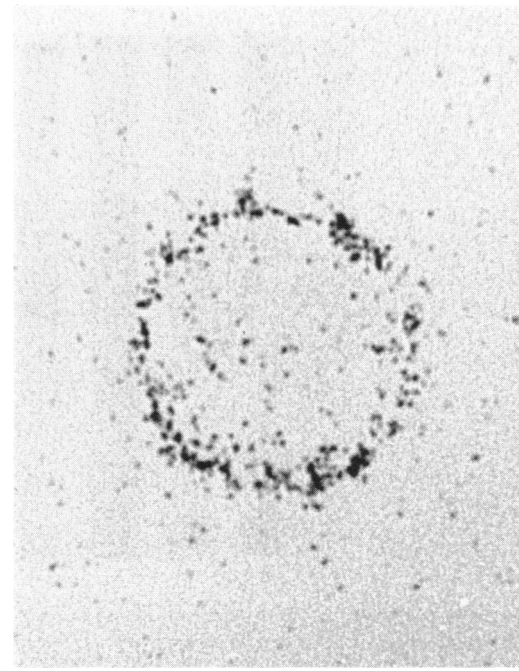

Fig. 2. Light microscope autoradiograph of a transverse section through a *S. ratti* adult worm radio-labelled using Iodogen. Similar appearances were seen with infective larvae radio-labelled using Iodogen.

Parasites labelled by each of the five methods were subjected to SDS-PAGE and the profiles of the labelled peptides were compared among the techniques and between the stages of the parasite. The profiles of infective larvae and adult worms labelled by choloramine T, lactoperoxidase and Iodogen are presented in Fig. 1. The three techniques resulted in very similar profiles within each stage of the parasite, while comparison between infective larvae and adult worms revealed stage-specific bands. The major labelled peptides of infective larvae had molecular weights of 130–160, 90, 56, 42 and 17 kD, while adult worms had major labelled peptides with molecular weights of 160, 17 and 14 kD. The SDS-PAGE analyses of parasites labelled with the Bolton and Hunter reagent or iodosulphanilic acid resulted in poorly-defined smears of peptides and are not shown. On the basis of these results, Iodogen was selected as the labelling method of choice as it provided the greatest amount of radioactivity bound to viable worms and the profile of labelled peptides was the same as that produced by less efficient techniques.

Surface location of radiolabelled peptides

The surface location of the isotope on Iodogen-labelled infective larvae and adult worms was confirmed by light microscope autoradiography of transverse sections of the worms. The location of silver grains indicated that ^{125}I was associated with the surface of the worm and there was no labelling of somatic structures (Fig. 2).

Solubilization of radiolabelled surface peptides

Once the parasites were iodinated, it was necessary to solubilize the labelled peptides for further analysis while still retaining their antigenicity. Four detergents were compared for their efficiency in releasing surface molecules from living parasites.

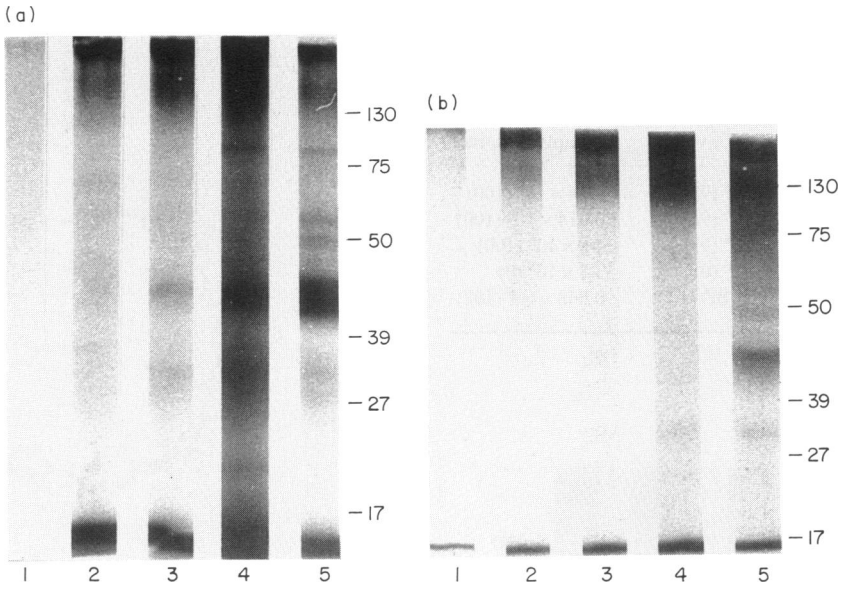

Fig. 3. SDS-PAGE analysis of detergent solubilization of Iodogen-labelled *S. ratti* infective larvae (panel a) and immunoprecipitation of these detergent-soluble extracts using hyperimmune mouse sera (panel b). Lane 1, antigen preparation buffer alone (APB). Lane 2, 1% Triton-X-100 in APB. Lane 3, 1% DOC in APB. Lane 4, 1% SDS in APB. Lane 5, 0·25% CTAB in APB. Molecular weight standards are indicated on the right (kD).

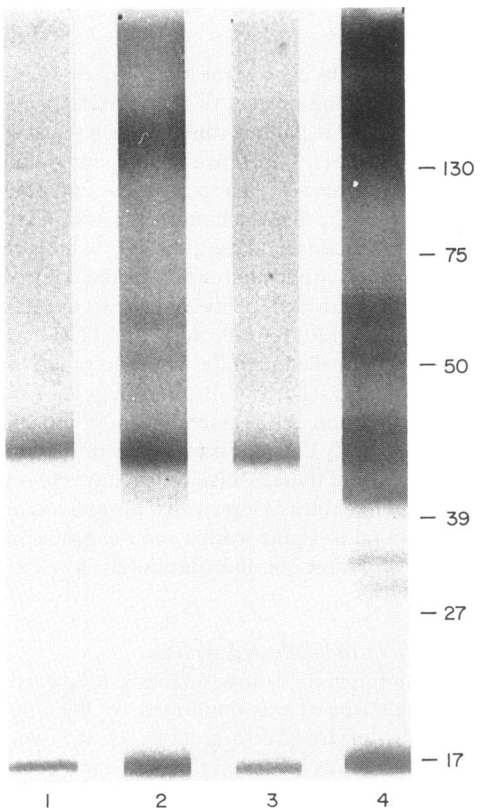

Fig. 4. SDS-PAGE analysis of immunoprecipitates formed between CTAB extracts of Iodogen-labelled *S. ratti* infective larvae and stage-specific mouse sera. Lane 1 normal mouse sera. Lane 2 sera from mice exposed to systemic infective larvae only. Lane 3 sera from mice exposed to intestinal adult worms only. Lane 4 sera from mice immunized by multiple complete infections. Molecular weight standards are indicated on the right (kD).

Panel (a) of Fig. 3 illustrates the SDS-PAGE analysis of the detergent-soluble fractions of infective larvae. The cationic detergent, CTAB, stripped more labelled material from the parasites than did either APB alone, Triton-X-100, or DOC, and yielded a cleaner preparation than did SDS. The results obtained from adult worms and infective larvae were similar.

Antigenicity of soluble surface peptides

The antigenicity of each detergent-soluble extract was assayed by immunoprecipitation with hyperimmune mouse sera raised by multiple, complete infections with *S. ratti*. The SDS-PAGE profiles of those immunoprecipitates formed with detergent extracts of infective larvae are illustrated in panel (b) of Fig. 3. The CTAB extract was superior among the detergent extracts in retaining antigenicity in the immunoprecipitation assay. Hyperimmune mouse sera precipitated bands with molecular weights of 52, 50, 44 and 32 kD and a smear of high molecular weight material from the CTAB extract. This was the largest number of bands recognized among the detergent extracts.

In contrast, no bands could be precipitated from any of the adult worm extracts.

Immunoprecipitation with stage-specific mouse sera

Attempts were made to establish whether there was any cross-reactivity between the recognition of adult and larval surface proteins by mice immunized with a single stage of infection. When CTAB extracts of infective larvae were reacted with normal serum, a non-specific single band of molecular weight 44 kD was precipitated (Fig. 4). Those mice exposed to an anthelmintic-abbreviated infection of infective larvae only also recognized this same band as well as a number of others at 160, 62, 52, 38 and 32 kD. Serum from mice exposed to adult worms in the intestine precipitated the non-specific band at 44 kD and no others. Mice immunized by multiple complete infections displayed the strongest reaction to larval CTAB extract; it was

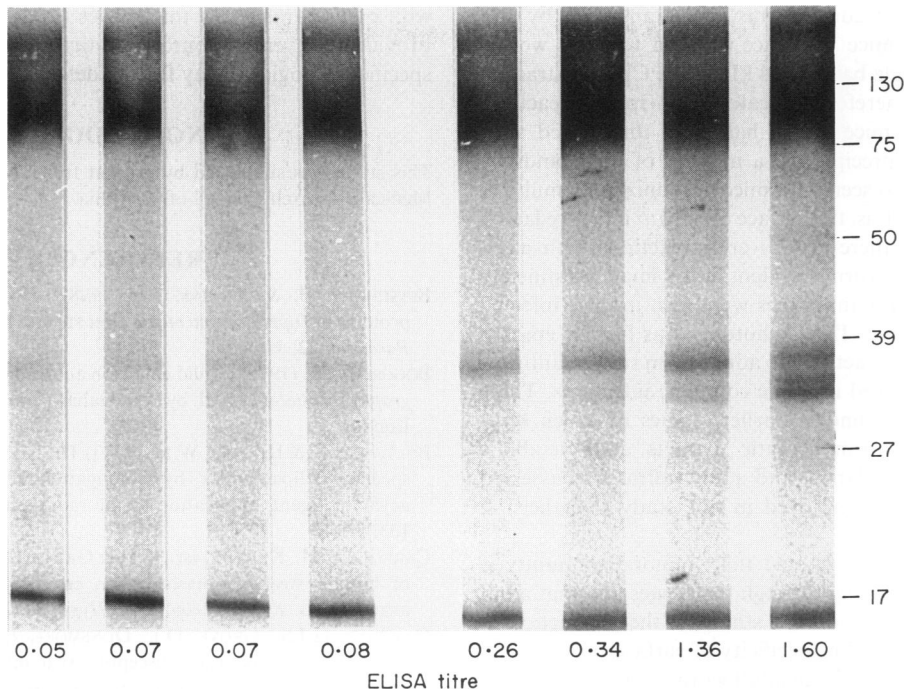

Fig. 5. SDS-PAGE analysis of immunoprecipitates formed between CTAB extracts of Iodogen-labelled *S. ratti* infective larvae and normal and infected human sera. ELISA values (optical density units) are indicated across the bottom and molecular weight standards are indicated on the right (kD).

similar to that of mice immunized with infective larvae with an additional band recognized at 29 kD. Again, no bands were precipitated from adult worm extracts by any of the mouse sera.

Immunoprecipitation with normal and infected human sera

Immunoprecipitation analysis of human sera from normal individuals and patients infected with *S. stercoralis* was performed against extracts of *S. ratti* infective larvae (Fig. 5). Two individuals with anti-*Strongyloides* ELISA titres of 1·36 and 1·60 optical density units (ODU) precipitated two bands of molecular weight 32 and 34 kD which were not recognized by normal sera. These bands were not seen in uninfected individuals but were discernable faintly in one of the two patients with borderline-positive serology.

DISCUSSION

The purpose of this study was to label and extract surface proteins of *S. ratti* infective larvae and adult worms in order that the antigens involved in the immune response of the host may be identified and characterized. Other studies have shown that it is necessary to examine a variety of labelling methods afresh for each nematode species or developmental stage under investigation (Philipp & Rumjaneck, 1984). The result of this comparison among labelling with chloramine T, lactoperoxidase, Iodogen, Bolton and Hunter reagent and iodosulphanilic acid was that iodination with Iodogen was the method of choice for this parasite. This agent produced the maximal binding of isotope to the surface of both stages of the parasite and resulted in no loss of viability. The specific surface location of the isotope was confirmed by autoradiography of transverse sections of worms. Iodogen has been successfully employed for the labelling of other nematodes e.g. *Dipetalonema viteae* (Betschart & Jenkins, 1987) and *Brugia malayi* (Marshall & Howells, 1985). In view of these findings, together with the observation of Philipp & Rumjaneck (1984), that the antigenicity of labelled components is better preserved with Iodogen-mediated reactions, Iodogen was chosen as the routine iodinating agent.

Conventional techniques for solubilization of antigenic surface molecules have employed non-ionic detergents such as Triton-X-100 or anionic detergents such as DOC or SDS to solubilize proteins from surface-labelled parasites. Recent work by Pritchard *et al.* (1985) has utilized the cationic detergent CTAB to strip antigens from the cuticle of *Nematospiroides dubius*. The potential value of this method for our study was suggested by the observation that the epicuticles of *S. ratti* and *Trichinella spiralis* both have a negative net surface charge and have been shown to bind the polyvalent cationic complexes, cationized ferritin and ruthenium red (Murrell & Graham, 1983). Our results proved that solubilization with CTAB was the method of choice as it yielded more labelled proteins than did APB, Triton-X-100 or DOC, and gave a more discrete profile than did solubilization with SDS. Furthermore, the greatest retention of antigenicity was achieved with solubilization using CTAB.

The existence of stage-specific surface components was apparent when the profiles of Iodogen-labelled infective larvae and adult worms were compared. The significance of this in relation to the location of the different stages of the parasite within the host and the host response to these parasites is not yet fully understood. In order to ascertain whether there was any cross-reactivity between the recognition of adult and larval surface proteins, we immunized mice with either a complete infection, infective larvae only (a drug-abbreviated infection

using cambendazole) or adult worms only (transferred by oral intubation). Normal mice and mice exposed to adult worms only recognized a single band at 44 kD in the CTAB extract of infective larvae: this therefore indicates a non-specific reaction. On the other hand, mice which had been immunized with infective larvae only precipitated a number of new bands. A similar profile was also seen with mice immunized by multiple complete infections. Thus, the surface of *S. ratti* infective larvae is immunogenic and there are no cross-reactions with adult worms. Further, adult worms resident in the small intestine are not immunogenic as no antibodies were seen in mice infected with adult worms alone. This dichotomy was further emphasized by the absence of reactivity to adult worm surface antigens by mice that had received multiple complete infections. These results parallel those found in earlier studies in which sero-reactivity to whole worm somatic extracts and metabolic products from infective larvae and adult worms was assessed using the same sera as employed in this study (Northern & Grove, 1988).

All of these studies suggested that humoral immunity is induced by larvae migrating through the tissues and that adult worms residing in the gut do not stimulate the production of serum antibodies. The stage-specificity of surface proteins and surprising lack of antigenicity of adult worms may play a role in the ability of adult worms to evade the immune response. The mechanism by which adult worms fail to generate serum antibody responses to surface components is uncertain. It is clear that the protein compositions of the surfaces of third-stage larvae and adult worms are different. It is likely that the antigenic profiles of the surfaces of infective larvae, fourth-stage larvae and adult worms vary. The immune system is exposed intimately to the surface of third-stage larvae during their migration through the tissues. On the other hand, fourth-stage larvae and adult worms are found only in the gut. Recent electron microscopical studies have shown that adult worms reside in tunnels among the enterocytes on the luminal side of the basement lamina (Grove *et al.*, 1987). Consequently, these stages of the lifecycle of the parasite are relatively isolated from the cells of the immune system and thus do not appear to generate systemic antibody responses.

We also sought to examine the existence of shared antigenic determinants between *S. ratti* and *S. stercoralis*. We used sera from normal individuals and from patients infected with *S. stercoralis*. Antibodies against *S. stercoralis* were measured in an ELISA. In this assay, values less than or equal to 0·20 ODU were considered negative, values between 0·20 and 0·40 ODU were regarded as borderline, while values greater than 0·40 ODU were considered positive.

The two individuals with titres of 1·36 and 1·60 ODU were strongly positive and precipitated two bands of molecular weight 32 and 34 kD. The individual whose ELISA titre was 0·34 ODU, regarded as a borderline case, also faintly precipitated these bands, whereas these bands were absent from the sera of the four uninfected persons. These results suggest the presence of potentially useful diagnostic antigens on the surface of *S. ratti* infective larvae. It remains to be shown, however, that these antigens are specific for *Strongyloides* and do not cross-react with other parasites. If this proves to be the case, *S. ratti* will provide a convenient source of antigen for the development of a specific serological assay for the detection of strongyloidiasis.

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