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The secreted and somatic antigens of the third stage larva of *Anisakis simplex*, and antigenic relationship with *Ascaris suum*, *Ascaris lumbricoides*, and *Toxocara canis*

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The in vitro-released 'excretory/secretory' (ES) and somatic antigens of the third stage (infective) larva of *Anisakis simplex* were characterised by radioiodination, immunoprecipitation, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Oral infection with the parasite elicited a substantial IgG antibody response to ES in infected rabbits and humans, with a minimal response to somatic materials. Serial serum sampling in experimental infection showed that there was a sequential recognition of distinct ES components. In contrast to oral infection, intraperitoneal exposure of rats with living parasites induced a strong response to both ES and somatic antigen preparations. Sequential recognition of ES antigens, and differential responses to somatic components, might, therefore, have application in the estimation of the age and degree of penetration by the nematodes in human infection. Extensive antigenic relationships were found between *A. simplex* and three other species of ascaridoid nematodes, namely *Ascaris lumbricoides*, *Ascaris suum*, and *Toxocara canis*, but none with a panel of non-ascaridoid nematodes. Evidence is presented that a M_r 14000 component of *A. simplex* has a homologue in all of the ascaridoids examined, but does not elicit an antibody response in anisakiasis. Finally, the ES of *A. simplex* is shown to contain two proteinase activities, of approximately M_r 23 400 and 46 100, as revealed by separation on gelatin substrate gels, although the antigenicity of the enzymes remains to be established.

Key words: *Anisakis simplex*; *Ascaris suum*; *Ascaris lumbricoides*; *Toxocara canis*; Nematode; Ascaridoidea; Antigen; Excretory/secretory antigen; Proteinase

Introduction

Infection of man with larval *Anisakis simplex*, commonly known as 'herringworm' or 'whale-

worm', and the accompanying acute gastro-intestinal symptoms, is becoming increasingly recognised as of public health concern in several countries [1–4]. Diagnosis of human infection is difficult in that it necessitates surgical investigation and/or fibre optic gastroscopy. The development of serodiagnostic assays is still in its early stages, and the best source of parasite antigen for use in assays has not been identified. Moreover, unlike other nematodes of the superfamily Ascaridoidea which infect man, little is known of the biochemistry and immunochemistry of *A. simplex* antigens. This presents limits to the development of immunological assays, particularly for a member of a group of nematodes such as the Ascaridoidea, within which there is probably considerable antigenic homology [5–7].

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Abbreviations: ABF, *Ascaris suum* pseudocoelomic fluid; ES, in vitro-released 'excretory/secretory' parasite products; DOC, sodium deoxycholate; L3, third stage larva; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

Here we have begun a characterisation of the secreted and somatic products of the developmental stage of the parasite which infects humans – the third stage larva (L3). The in vitro-released (excretory/secretory or 'ES') materials of L3 have been used for serodiagnostic purposes [8], and are known to contain proteinases [9,10]. We find that the ES is the subject of a substantial IgG antibody response in human anisakiasis and in experimental animal infections, and that its constituents are recognised sequentially during the development of the immune response. Somatic antigens elicit a considerably less marked response, but might be of relevance to serological determination of the degree of penetration, moulting, and/or parasite death within the tissues of the opportunistically infected human host.

The Ascaridoidea present an excellent opportunity to study the principles of antigenic homology between nematode parasites in general, providing a conveniently obtained and well-defined set of parasites. Here, this was examined using sera from defined infections of rabbits with three species of ascaridoids which are of relevance to man, namely, *Toxocara canis*, *Ascaris lumbricoides*, and *Ascaris suum*. This brought to light a somatic and secreted constituent of *A. simplex* which might have a homologue in all ascaridoid nematodes.

Materials and Methods

Parasite products. *A. simplex* third stage larvae (L3) were collected by dissection from herring (*Clupea harengus*) caught in the northern North Sea. The geographical origin of the parasites used is consistent with them belonging to the sibling species *A. simplex* B, which has been electrophoretically distinguished from the sibling species *A. simplex* A (Mediterranean Sea) [11]. Viable larvae were selected and cultured at approximately 5 larvae ml⁻¹ in the following protein-free medium which is in use for culture of *Ascaris* larvae [12]: RPMI 1640, Dutch modification (Flow Laboratories, Irvine, product number 12-609-54), containing 1 mM sodium pyruvate, 400 ng ml⁻¹ glycyl-L-histidyl-L-lysine (Sigma G 1887), 16 µM glutathione (reduced, Sigma 4251), 1 mg ml⁻¹ glucose, 100 I.U. ml⁻¹ penicillin, 100 µg ml⁻¹

streptomycin. Only larvae which were microscopically free of host tissue were used, and those selected were cultured for 24 h to further reduce contamination with host materials before culturing for the production of in vitro-released materials (ES). The cultures were maintained at 37°C in 5% CO₂, the medium harvested and replenished every 2 days for 8 days, then pooled. ES was filtered through a 0.22 µm, low protein-retention filter (Millex GV SLGV025BS; Millipore S.A., Molsheim, France), and stored at -70°C until used. *A. suum* in vitro-released material was collected following previous methods [5,12].

A. simplex larvae were homogenised, after at least 24 h of culture, in a glass tissue homogeniser, in 10 mM Tris buffer, pH 8.3, with and without 1% sodium deoxycholate (Sigma D 6750), containing the following proteinase inhibitors: 2.0 mM ethylenediaminetetraacetic acid (Sigma ED3SS), 1.0 mM phenylmethylsulphonyl fluoride (Sigma P 7626), 5 µM pepstatin A (Sigma P 4265), 2 mM 1,10 phenanthroline (Sigma P 9375), 5 µM leupeptin (Sigma L 2884), 5 µM antipain (Sigma A 6271), 25 µg ml⁻¹ *N*-p-tosyl-L-lysine chloromethyl ketone (Sigma T 7254), and 50 µg ml⁻¹ *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma T 4376). Homogenisation was performed over ice and checked for completion by microscopy. The homogenate was centrifuged at 13 000 × *g* for 20 min in a microfuge, and the supernatant collected.

Homogenate supernatants and ES were snap frozen in liquid nitrogen, and stored at -70°C until used.

Antisera. A rabbit (Sandy Lop; Hylyne Rabbits Ltd., Northwich, Cheshire) was serum sampled then infected by stomach tube with 20 larvae of *A. simplex* on days 0, 14, and 28, and bled serially thereafter. Serum to this parasite was also raised in (Lister × BN)F₁ rats exposed intraperitoneally by laparotomy with 5, 10, or 15 larvae. Lister rats are outbred, and BN rats inbred. Serum from a patient (AM) with a confirmed infection with *A. simplex* [8] was obtained from the Scottish Parasite Diagnostic Laboratory, Stobhill Hospital, Glasgow. Antiserum to *A. suum* was raised in rabbits infected every 28 days with 3000 embryonated eggs of the parasite, and serum col-

lected 14 days following the last of four infections. Antiserum to the pseudocoelomic fluid (usually termed 'Ascaris body fluid', and abbreviated 'ABF') of *A. suum* was raised in rabbits using a conventional immunisation protocol with Freund's adjuvant. Briefly, one injection of 1 mg protein in Freund's complete adjuvant was given into the muscle of one thigh, followed seven days later by a similar injection into the thigh muscle of the opposite leg. Two months later, the rabbit was boosted with the same amount of antigen presented in Freund's incomplete adjuvant distributed among five subcutaneous sites on the back, and bled for serum on days 14, 17, 19, and 21 later. Antibody to the M_r 14 000 component of *A. suum* was prepared as previously described using material excised from polyacrylamide gels [5]. Anti-*T. canis* serum was collected from a rabbit infected with 20 000 viable eggs of the parasite, and bled 56 days later, on the plateau of the serum antibody response. Serum was also collected from rats infected with *Nippostrongylus brasiliensis* or *Strongyloides ratti*, and from mice infected with *Trichinella spiralis* or *Trichuris muris*.

Radio-iodination. Dialysed culture medium or worm homogenate was labelled with ^{125}I using the IODO-GEN method [13] following previous methods [12]. The isotope was obtained from the West of Scotland Radionuclide Dispensary or Amersham International, and the iodination reagent bought from Pierce Chemical Co., Rockford, IL, U.S.A. (28600). The amount of protein labelled was approximately 2 μg and 20 μg for ES and homogenate, respectively. The body fluid of adult *A. suum* worms was collected as previously described [5,12], dialysed against phosphate buffered saline (PBS), and 20 μg protein radiolabelled as above.

Radio-immunoprecipitation. This was performed using a *Staphylococcus aureus*-based assay [5,12] in which iodinated culture medium was incubated with serum overnight at 4°C, and IgG immune complexes captured on heat-killed, formalin-fixed bacteria (Pansorbin Standardised 507861; Calbiochem-Behring, La Jolla, CA, U.S.A.). Radioactivity bound was counted in a gamma counter and

the pellets prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE. This was carried out in a Pharmacia (Uppsala, Sweden) GE-2/4 LS slab gel apparatus according to the manufacturer's instructions. Gradient gels (5–25%) consisted of 120 mm separating gel and 15 mm stack gel, and were 0.7 mm thick. Samples were prepared according to published methods [14], with 5% mercaptoethanol or 1 mg ml⁻¹ iodoacetamide for reducing and non-reducing conditions, respectively. Following electrophoresis, gels were fixed in 25% methanol, 10% acetic acid, and 1% glycerol, then dried. Autoradiographs were exposed at -70°C with flashed Fuji RX X-ray film. Apparent molecular masses were estimated with reference to standard marker proteins (Pharmacia 17-0446-01).

Proteinase detection in SDS-PAGE. Proteinases in helminth culture fluid were detected using established techniques [15–18]. Briefly, gelatin (0.1% final concentration) (bacteriological grade, 152-0221; Gibco, Europe Ltd., Paisley, Scotland) was copolymerised with 5–25% gradient polyacrylamide gels. Culture supernatants were brought to approximately 20 times starting concentration, in centrifugal ultrafiltration microconcentrators with a nominal molecular mass cut off of 10 000 ('Centricon 10', 4205; Amicon Ltd., Stonehouse, U.K.). A 15 μl sample of this was mixed with an equal volume of SDS-PAGE sample buffer and applied to a gel without boiling of the sample. The standard SDS-PAGE sample buffer was modified to contain 20% glycerol and no proteinase inhibitors. The gels were run at a high current (60 mA per gel), to allow a short separation time, and the electrophoresis tanks were pre-cooled and maintained in a 4°C environment, to reduce enzyme activity during electrophoresis. On completion of the run, gels were removed from their cassettes, rocked in 2.5% Triton X-100 for 20 min, washed in three changes of distilled water over 20 min, and incubated without disturbance overnight in 0.1 M Tris buffer pH 7.5 at 37°C. Gels were then fixed, stained in Coomassie blue (Brilliant Blue R 250, Sigma B 0630), destained, and dried onto cellophane film for photography. Elastase (EC 3.4.21.36, Sigma

E 0258) was used as a positive control, whose molecular weight is 25 900. It was established, using radiolabelled enzyme, that the M_r of this marker was not affected by boiling or reduction in the electrophoresis system used here.

Results

The somatic and secreted components of A. simplex. SDS-PAGE analysis of the *A. simplex* ES components which can be radiolabelled using the 1,3,4,6-tetrachloro-3a,6a-diphenyl glycouril reagent is given in Fig. 1A. ES was found to contain components over the range of M_r 11 000–223 000 with faint bands greater than this, which is a wider range than previously described [19]. The highest M_r molecules, those over M_r 130 000, were reduction labile, and by running reduced and non-reduced samples of ES in adjacent tracks, a shift in mobility of several other components was observed. The lateral diffusion of the reducing agent during electrophoresis produced molecules in a transition state in the adjacent track, generating diagonal lines connecting the positions of some molecules under each condition. Thus, a component of M_r approximately 43 000 was seen to decrease its mobility after reduction, an effect also apparent with some of the molecular weight standards used here. All the components of ES were proteinaceous as defined by susceptibility to degradation by pronase and proteinase K (not shown).

The use of proteinase substrate gels revealed two bands of proteinase activity on gelatin at pH 7.5 (Fig. 1B) from ES, the M_r of which we estimate to be 23 400 and 46 100. We attempted to relate bands in autoradiographs of radio-iodinated ES, or immunoprecipitations thereof, to the positions of the zones of proteolysis, but were unable to obtain a precise correlation. This failure was compounded by the loss of radiolabelled materials during the overnight incubation of the zymograms, but it is also possible that the enzymes are poorly iodinated, low in concentration in comparison to other components, or migrate at a different rate under conventional SDS-PAGE conditions. However, with respect to the latter possibility, the migration of both radiolabelled elastase marker and the major radioiodinatable

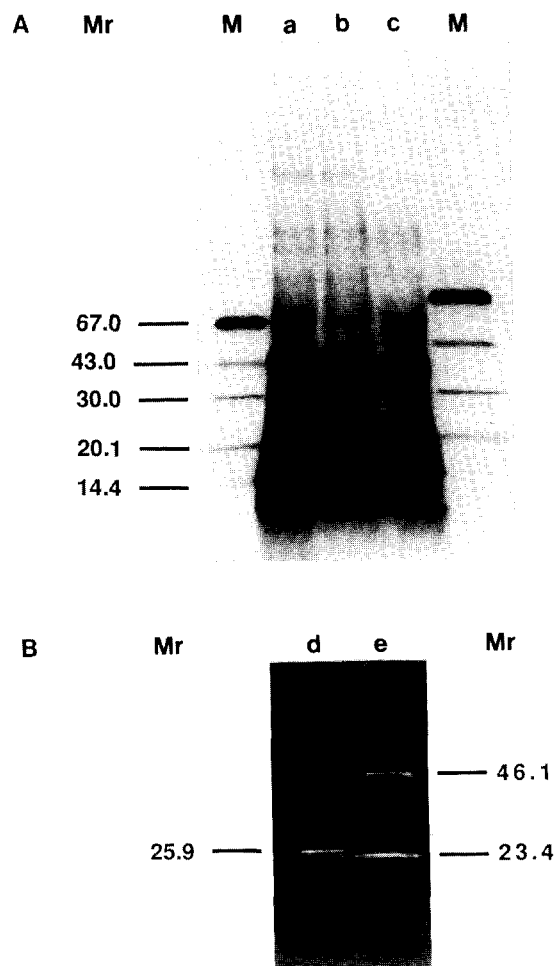


Fig. 1. (A) Gradient SDS-PAGE profile of in vitro-released (ES) products from *A. simplex* third stage larvae. Culture fluid was labelled with the IODO-GEN reagent, and electrophoresed on 5–25% gradient gels under non-reducing (a,b) and reducing (c) conditions. The lateral diffusion of reducing agent from track c produced partial reduction of the molecules in track b, permitting the tracing of alterations of M_r of some molecules brought about by reduction. Radioiodinated molecular weight standards (Pharmacia 17-0446-01) were run in the lateral tracks (M), the right hand one reduced, and their nominal M_r , as given by the manufacturer ($\times 10^3$). (B) SDS-PAGE separation of proteinase activities in *A. simplex* ES. Concentrated culture supernatant (e) was electrophoresed down gradient gels which had been co-polymerised with gelatin as proteinase substrate, then incubated overnight at 37°C at pH 7.5. The zones of proteolysis were revealed by staining the gel with Coomassie blue. Track (d) shows the position of elastase run on the gel under identical conditions. Molecular weights were estimated from a standard curve using the same protein standards as used above.

ES components was not affected by boiling (not shown).

Although not illustrated at this point, the SDS-PAGE profiles of ^{125}I -labelled worm homogenates made in the presence and absence of 1% sodium deoxycholate (DOC) showed that detergent extraction did not appear to increase the yield of the major constituents, nor did it lead to extra bands on the autoradiographs. Reduction of homogenate led to the loss of the highest molecular mass components, those over M_r 185 000, but we have made no attempt at this stage to identify the products of this reduction.

Sequential recognition of secreted antigens during infection, and the human antibody response. The specificity of the antibody responses of rabbits given multiple oral infections with the infective larvae of *A. simplex*, a human who contracted anisakiasis in the U.K. [8], and rats infected with the parasite intraperitoneally, were examined. In the rabbit (Fig. 2), serial serum sampling revealed that the recognition of the components of

ES was sequential. Although not shown in the experiment illustrated, the recognition profiles of sera taken after day 63 showed a sequential loss of precipitation of the components, the major band at approximately M_r 20 000 being among the first to disappear. This decay would imply that the infection was self-limiting in the rabbit, as was found to be the case at autopsy. The amplitude of the IgG response to the ES is shown to better advantage in Fig. 3.

The antibody response of the infected human (Fig. 2, track k), like the rabbit, precipitated most of the components of ES, with the notable exception of the M_r 20 000 molecule. It could be inferred from the rabbit data that this might be due to serum sampling either during the development of the humoral response, or during its decay. The clinical information [8] would infer that the former is the case, although it is also possible that there is a genetic restriction of antigen recognition [20].

It is known in human anisakiasis that penetration and perforation of gastrointestinal tissue can

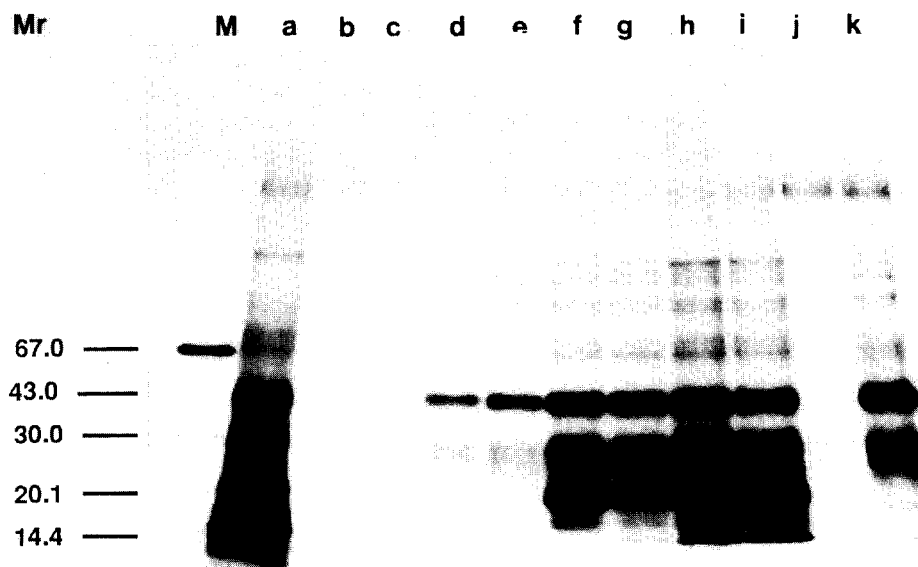


Fig. 2. Antigenicity of *A. simplex* ES proteins in the context of oral infection in man and rabbits. ^{125}I -labelled *A. simplex* ES (a) was immunoprecipitated in a protein A-based assay with serum from a rabbit infected orally with 20 *A. simplex* larvae on each of days 0, 14, and 28. The rabbit was bled on the following days: pre-infection (b), 7 (c), 21 (d), 28 (e), 35 (f), 42 (g), 58 (h) and 63 (i). The recognition profile of 'normal' human serum (j) and serum from a human patient with surgically confirmed anisakiasis (k) was analysed in the same immunoprecipitation experiment as the rabbit sera. M, marker proteins.

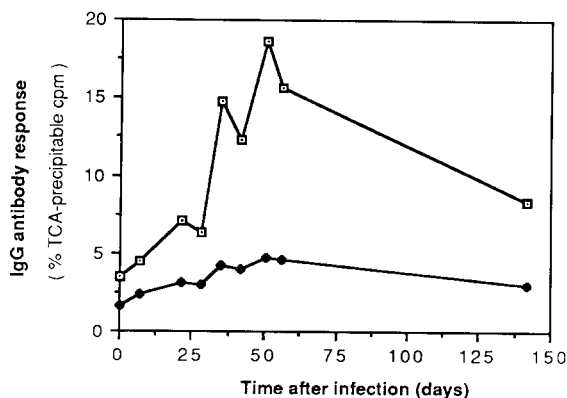


Fig. 3. Kinetics of the IgG antibody response in the rabbit following serial infection with *A. simplex*. A rabbit was orally infected with 20 larvae on each of days 0, 14, and 28, and blood samples were taken on the days indicated. Radiolabelled ES (□) or TRIS homogenate (◆) of the parasites were immunoprecipitated in the protein A-based assay, as described in Materials and Methods. Results are expressed as the mean percentage TCA-precipitable cpm per assay tube, and each point represents the mean from three separate experiments.

occur [1–4]. The antibody response to such an occurrence was, therefore, modelled by exposing rats to the parasite by direct introduction of living larvae into the peritoneal cavity. This generated a strong antibody response to internal components of the parasite (see below), but also elicited a substantial response to ES (Fig. 4). The rats were autopsied at 63 days post infection, at which time none of the worms remained alive, and residual material, of presumably both parasite and host origin, was found close to the site of laparotomy. This inferred that the infection was either short lived, or rapidly confined by the host. The recognition profiles appeared to vary between individual rats, but this did not relate to the dose of parasites given, and might relate to the major histocompatibility complex haplotype of the hosts [20]. Similar heterogeneity in responses was found using homogenate as target antigen (see below).

Anisakis ES and antigenic relationships with *Toxocara* and *Ascaris*. It is already established that radioimmunoassay combined with SDS-PAGE can detect considerable antigenic cross-reaction between ascaridoid nematodes which infect man [5–7]. It was, therefore, pertinent to examine this

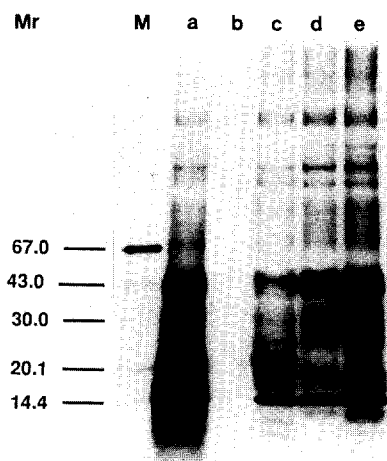


Fig. 4. The *A. simplex* ES components recognised by serum IgG antibody raised in exposure to living larvae by the intraperitoneal route. Radioiodinated ES (a) was immunoprecipitated with normal rat serum (b), or serum from rats given 5 (c), 10 (d) or 15 (e) larvae intraperitoneally by laparotomy, and bled 28 days later. M, marker proteins.

with *A. simplex* for two reasons. First, as a nematode of this group, it could potentially interfere with immunoassay for related parasites, and vice versa. Secondly, having a multi-host marine life-cycle rather than a direct terrestrial one [21], it might be expected to be antigenically remote from ascaridoids such as *Toxocara* spp. and *Ascaris* spp. The results of a cross-reactivity screening is given in Fig. 5 in which sera raised against examples of the latter genera, and some non-ascaridoid nematodes were used. Of particular note is the virtually complementary recognition profiles of the anti-*Ascaris* and anti-*T. canis* sera (Fig. 5A). In contrast to this extensive heterologous recognition, Fig. 5B testifies to the lack of similar interaction using serum raised against a panel of non-ascaridoid nematodes. The antibody responses elicited by the various ascarid infections differed notably against ES molecules in the M_r 14 000 region of the gels. While both the anti-*A. simplex* and anti-*Ascaris* sera precipitated molecules of this approximate mass, the anti-*T. canis* serum did not. Also, there appeared to be a slight disparity between the migration of the dominant component precipitated by the former two sera. Secretion by *A. simplex* of a molecule related to

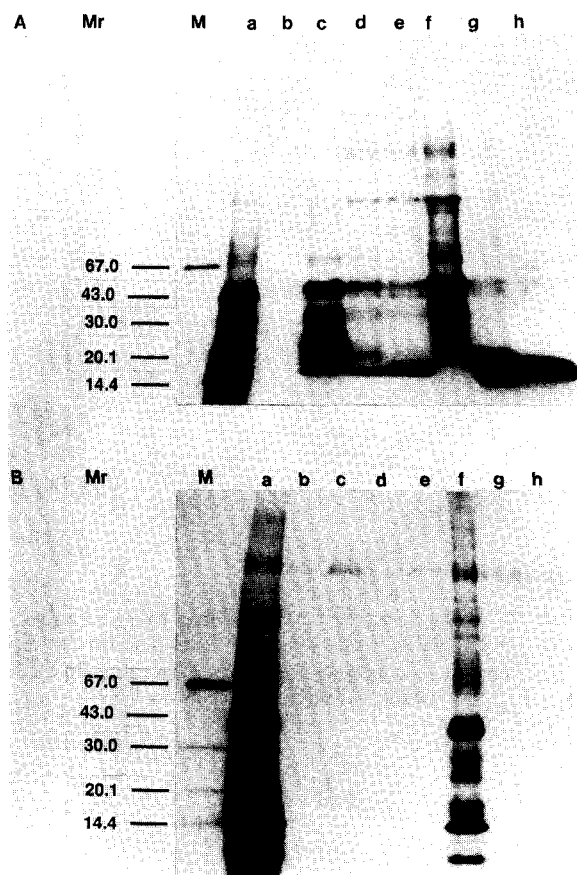


Fig. 5. Antigenic relationship between *A. simplex* and other ascaridoid nematodes. (A) Immunoprecipitation of radioiodinated *A. simplex* ES (a) with normal rabbit serum (b), and serum from rabbits infected orally with *A. simplex* (c), *A. suum* (d), *A. lumbricoides* (e), *T. canis* (f), or immunised with *Ascaris* body fluid (g) or the M_r 14000 component thereof in adjuvant (h). (B) Immunoprecipitation of radioiodinated *A. simplex* ES (a) with normal mouse serum (b), serum from mice infected orally with *T. spiralis* (c), mice infected with *T. muris* (d), normal rat serum (e), rats infected with 10 *A. simplex* larvae intraperitoneally (f), *N. brasiliensis* (g), or with *S. ratti* (h). M, marker proteins.

the *Ascaris* M_r 14000 molecule was inferred using a rabbit polyclonal antibody specific for this molecule (Fig. 5A, track h). *A. simplex*, then, might synthesise a homologue of the *Ascaris* M_r 14000, and infection did not elicit an IgG antibody response to it.

Specificity of antibody responses to somatic parasite materials. Oral infection in the rabbit generated a response in which only a somatic antigen of M_r 46000 was strongly precipitated (Fig. 6, track c). Intraperitoneal exposure, on the other hand, led to a humoral response with specificity for a much greater range of molecules (Fig. 6, tracks i,j,k), and, as for ES, the recognition profiles varied from host to host, in a manner which did not relate to parasite dose. This heterogeneity might, therefore, be due to genetic differences in the host, as has been established for *Ascaris* infection in rodents [20], and might apply to other nematode antigens in the context of infection [22]. With regard to the relationship between ES and somatic materials, it is noteworthy that there is some similarity in the pattern of bands produced in the 43 kDa region of gel immunoprecipitates of ES and homogenate by the individual rats (Figs. 4 and 6).

The anti-*Ascaris* reagents also precipitated *A. simplex* homogenate components, and the analysis also revealed some fine specificities of these antisera. For instance, whereas infection of both humans and rabbits with *A. simplex* elicited a response to the M_r 46000 somatic component, the anti-*Ascaris* preparation precipitated a slightly smaller molecule. Although not shown here, anti-*T. canis* infection serum precipitated the M_r 46000 component and not the lesser. These differential reactivities would indicate, therefore, that the two molecules are not related in spite of their similar appearance and M_r on the autoradiographs.

Lastly, as was also apparent for *A. simplex* ES, antiserum raised to ABF, and an antibody specific for the M_r 14000 molecule thereof, precipitated a component in *A. simplex* homogenate of this M_r (Fig. 6, track e). In contrast, neither the oral nor the intraperitoneal *Anisakis* infection serum precipitated a molecule of this size (Fig. 6, tracks c,g,i,j,k).

A common M_r 14000 component of ascaridoid nematodes? Given the already established importance of the M_r 14000 component of *Ascaris* in the immunology of infection with this parasite [5,12,20], it was pertinent to further investigate its homology with that of other ascaridoids. In the final two experiments, the relationship was inves-

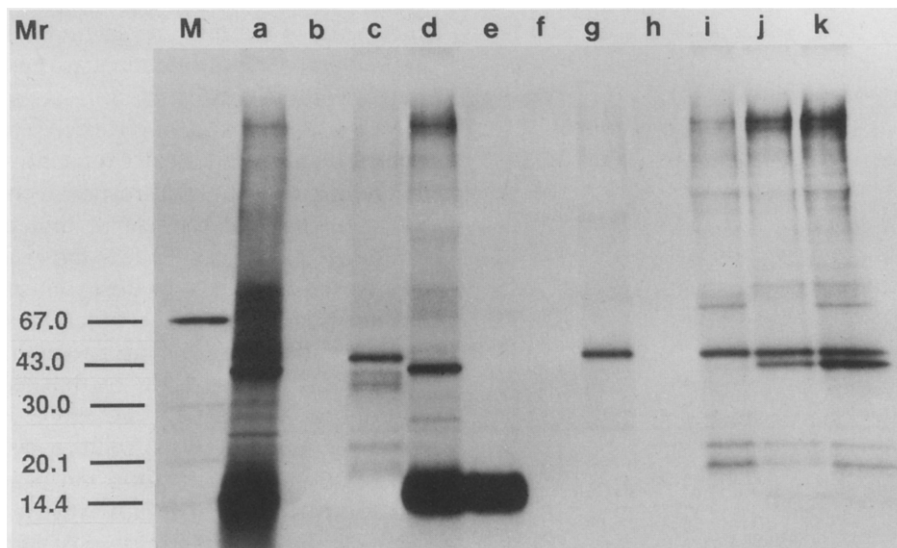


Fig. 6. *A. simplex* has a somatic component which is probably a homologue of the M_r 14000 antigen of *Ascaris*, and is not a target of the antibody response in infected rabbits, rats, or humans. Radioiodinated TRIS homogenate of *A. simplex* (a) was immunoprecipitated with normal rabbit serum (b), serum from a rabbit infected with *A. simplex* (c), or rabbits immunised with *Ascaris* ABF (d) or the M_r 14000 molecule thereof (e), normal human serum (f), a human infected with *A. simplex* (g), normal rat serum (h), or rats infected with 5 (i), 10 (j), or 15 (k) larvae intraperitoneally. M, marker proteins.

tigated using radio-iodinated *Ascaris* body fluid as target antigen. Given the probable non-recognition of the homologue elicited by anisakiasis, immunoprecipitation was first carried out using an antiserum made in rabbits to *A. simplex* homogenate presented in adjuvant (Fig. 7). In this case, the *Ascaris* M_r 14000 was clearly precipitated, as was also found to occur when the target antigen was radio-iodinated ES of *A. suum* lung-stage larvae, which also contains the M_r 14000 (not shown). When a similar experiment was carried out using serum raised in the context of either rabbit oral, or rat intraperitoneal, infection, the M_r 14000 was not precipitated (not shown) as also was found with anti-*T. canis* infection serum. It could be concluded, then, that *A. simplex* did indeed contain an *Ascaris* M_r 14000 homologue, but that infection did not appear to elicit a response to it, and, surprisingly, this applied even under conditions of intraperitoneal exposure.

Discussion

The materials released by the tissue-invasive larval stages of species from three ascaridoid ne-

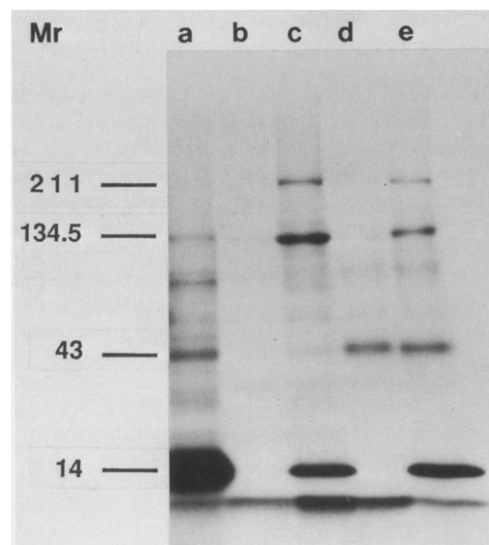


Fig. 7. Antigenic cross-reactions between *Ascaris* and *A. simplex* somatic materials. Radioiodinated *Ascaris* body fluid (a) was immunoprecipitated with normal rabbit serum (b), or serum from rabbits infected with *A. suum* (c), *T. canis* (d), or immunised with homogenate of *A. simplex* (e). M, marker proteins.

matode genera infective to man have now been characterised. Each species secretes a set of proteins and/or glycoproteins with unique SDS-PAGE profiles, although there are distinct similarities within a genus [5–7]. Moreover, the ascaridoids clearly show extensive antigenic relationships in spite of their diversity in life cycles and hosts. This has been examined at the epitope level only for *T. canis* and *Toxocara cati*, in which it has been found that most MAbs raised to the ES of the former recognise several components from the ES of the latter. Moreover, at least one epitope is unique to *T. canis* [6,7], and this is a glycosylation which occurs on several distinct secretory products [6,23]. We have no information at present on the extent of glycosylation of the ES of *Anisakis*, but it would clearly be instructive to understand the role of carbohydrate epitopes in the antigenic cross-reactions which we find to be widespread within the Ascaridoidea.

It has been argued that surface and secreted antigens of helminth parasites, because they comprise the host–parasite interface, are of greater relevance to an understanding of host–parasite interaction than are internal components [24]. Moreover, ES products are increasingly preferred as a source of diagnostic and protective antigens [25]. For anisakiasis, our present results would add weight to this, for radioimmunoassay at least. This was emphasised by the SDS-PAGE analysis which showed that a greater number and proportion of components were recognised from ES than from homogenates.

The response to internal molecules might nevertheless be clinically and seroepidemiologically relevant as an indicator of exposure to internal components at parasite penetration into host gastrointestinal tissue, and/or during its moulting or following its death. In such a situation, therefore, worm somatic antigens might be of relevance to immunopathological reactions. Modelling tissue penetration by intraperitoneal exposure of rats to living larvae led to a much greater response to internal components than did oral infection, but it also generated a substantial response to ES. This last finding could indicate that the parasites survived for sufficient time to stimulate a response to ES before their demise, or that the endogenous store of ES materials was

sufficiently large in itself to stimulate the immune system following disintegration of the worms.

In spite of the manifest antigenicity of ES components, the rabbit IgG antibody response to infection displayed a distinct sequential recognition of ES antigens. Such an effect has been noted in the recognition of surface antigens of *Trichinella spiralis* [26], and ES of *Ascaris* [5,20], although we find that it is not the case in experimental infection with *T. canis* (Ruddy, M. and Kennedy, M.W., unpublished observations). For anisakiasis, it remains to be seen whether this is due to differential development of different antibody specificities, or the sequential release of the various secretory components by the parasite in vivo.

A case has been made for the use of an IgE-based serological assay for anisakiasis based on somatic antigens [1]. This arose because of the apparent paucity of the specific IgG response, yet, presumably because of the nature of the antigen itself, or the manner of its presentation to the host, a specific IgE response is detectable. On the other hand, we find that an IgG response to ES in rabbits, rodents, and man is readily stimulated, which would argue for the use of this antigen source in serodiagnosis. A similar case for an IgE-based assay has been made for the serological detection of *Ascaris* infection [27,28], although IVRs from the tissue-invasive larval stages of this parasite are now known to be subjects of a substantial IgG antibody response [5,12].

The cross-reactivity we have observed with *A. simplex* ES would clearly be of concern in the development of immunodiagnostic assays. However, this also applies to worm somatic materials, and it remains to establish whether or not the antigenic cross-reactivity identified by our methods will significantly affect the specificity of other immunoassays, for example, enzyme-linked immunosorbent assay (ELISA). Radioimmunoprecipitation might be over-sensitive in the detection of cross-reactions since only one epitope on a given molecule is theoretically sufficient for its inclusion in an immunoprecipitate, whereas ELISA is more quantitative of the number of antibody binding sites.

Among the cross-reactive antigens identified in this study was one for which there was good evidence that a homologue is synthesised by all the

ascaridoids dealt with here. This M_r 14000 molecule is the most abundant protein of the perienteric fluid of *Ascaris* [12], and an immunologically cross-reactive molecule of identical M_r is also found in perienteric fluid of *T. canis* (M.W.K., unpublished). A component of the same molecular mass was identified in both the ES and somatic compartments of *A. simplex* using a specific antibody to the M_r 14000 of *Ascaris*. Unexpectedly, however, infection with *Anisakis* did not generate an antibody response to the M_r 14000 of the homologous parasite, in contrast to infection with *A. suum* and *A. lumbricoides* [5,12]. In this respect, then, anisakiasis might resemble toxocariasis [5].

We cannot explain this apparent nonrecognition of a secreted and somatic antigen, but several possibilities arise. First, oral infection with *A. simplex* might not expose the host to this component in sufficient quantity to generate an antibody response. However, it would be difficult to reconcile this with the lack of a response in rats exposed intraperitoneally with larvae which were subsequently killed and broken down. Secondly, it is possible that the hosts used here were genetically incapable of responding to this antigen in the context of infection, as is known to occur in *A. suum* infection in mice [20]. On the other hand, the rabbit was chosen as a responder to the M_r 14000 of *A. suum* in the context of infection [12]. Final possibilities lie in differences in the immunochemistry of the M_r 14000 from different species, and/or subtle differences in the component in its secreted or somatic forms.

We have not found a similar molecule among a range of other parasitic nematodes, and conclude that the M_r 14000 could be a characteristic of the Ascaridoidea. The members of this superfamily, however, seem to produce it in different quantities, expose their hosts to it differently, and the details of the immunochemistry of the molecule might vary from species to species. Nevertheless, an antibody raised to the purified *Ascaris* molecule detects components of the same M_r from all the nematodes studied here, and might also do so for other zoonotic ascaridoid infections of humans.

Several parasitic nematodes are known to release proteinases in vitro, including the three as-

caridoid species considered here, *T. canis* [29], *A. suum* [30], and *A. simplex* [9,10]. Our present results show that there are two activities in *A. simplex* ES, in terms of migration in SDS-PAGE, and that these are active at pH 7.5 and 37°C. We have not established the temperature optimum of these enzymes, but their stimulated release [9] and activity at mammalian body temperature would seem appropriate for entry into the definitive, and the paratenic human, host.

The M_r of these enzymes in *A. simplex* we estimated to be 23400 and 46100, and there could be, therefore, a monomer-dimer relationship between them. Without more detailed information on the substrate specificities and inhibitor sensitivities of the two, we cannot as yet draw any firm conclusions about their relationship. We have also not established the antigenicity of the enzymes in the context of infection, but there are major bands in the appropriate region of autoradiographs of radiolabelled and immunoprecipitated ES material (Fig. 2). If these are indeed the proteinases, then they appear to be sequentially recognised during the development of the antibody response to infection in rabbits, and the smaller is not recognised by the human serum at the time of its sampling in the case examined. If the enzymes are involved in tissue invasion and pathogenesis then it would be particularly relevant to establish whether or not they can be inhibited by host effector mechanisms. With respect to this, the ES proteinases of tissue invasive larvae of *A. suum* are inhibited by antibody raised in the context of infection [30].

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References

- 1 Desowitz, R.S., Raybourne, R.B., Ishikura, H. and Kliks, M.M. (1985) The radioallergosorbent test (RAST) for the serological diagnosis of human anisakiasis. *Trans. R. Soc. Trop. Med. Hyg.* 79, 256–259.
- 2 Sugimachi, K., Inokuchi, K., Ooiwa, T., Fujino, T. and Ishii, Y. (1985) Acute gastric anisakiasis: analysis of 178 cases. *J. Am. Med. Assoc.* 253, 1012–1013.
- 3 Oshima, T. and Kliks, M. (1986) Effects of marine mammal parasites on human health. In: *Parasitology: Quo vadis? Proceedings of the Sixth International Congress of Parasitology, Brisbane, 24th–29th August, 1986*, pp. 415–421.
- 4 Oshima, T. (1987) Anisakiasis – is the Suchi bar guilty? *Parasitol. Today* 3, 44–48.
- 5 Kennedy, M.W., Qureshi, F., Haswell-Elkins, M. and Elkins, D.B. (1987) Homology and heterology between the secreted antigens of the parasitic larval stages of *Ascaris lumbricoides* and *Ascaris suum*. *Clin. Exp. Immunol.* 67, 20–30.
- 6 Maizels, R.M., Kennedy, M.W., Meghji, M., Robertson, B.D. and Smith, H.V. (1987) Shared carbohydrate epitopes on distinct surface and secreted antigens of the parasitic nematode *Toxocara canis*. *J. Immunol.* 139, 207–214.
- 7 Kennedy, M.W., Maizels, R.M., Meghji, M., Young, L., Qureshi, F. and Smith, H.V. (1987) Species-specific and common epitopes on the secreted and surface antigens of *Toxocara cati* and *Toxocara canis* infective larvae. *Parasite Immunol.* 9, 407–420.
- 8 Watt, I.A., McLean, N.R., Girdwood, R.W.A., Kissen, L.H. and Fyfe, A.H.B. (1979) Eosinophilic gastroenteritis associated with a larval anisakine nematode. *Lancet* 2, 893–894.
- 9 Matthews, B.E. (1982) Behaviour and enzyme release by *Anisakis* sp. larvae (Nematoda: Ascaridida). *J. Helminthol.* 56, 177–183.
- 10 Matthews, B.E. (1984) The source, release and specificity of proteolytic enzyme activity produced by *Anisakis simplex* larvae (Nematoda: Ascaridida) in vitro. *J. Helminthol.* 58, 175–185.
- 11 Nascetti, G., Paggi, L., Orecchia, P., Smith, J.W., Mattiucci, S. and Bullini, L. (1986) Electrophoretic studies on the *Anisakis simplex* complex (Ascaridida: Anisakidae) from the Mediterranean and North-East Atlantic. *Int. J. Parasitol.* 16, 633–640.
- 12 Kennedy, M.W. and Qureshi, F. (1986) Stage-specific secreted antigens of the parasitic larval stages of the nematode *Ascaris*. *Immunology* 58, 515–522.
- 13 Markwell, M.A.K. and Fox, C.F. (1978) Surface-specific iodination of membrane proteins of viruses and eukaryotic cells using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochemistry* 17, 4807–4817.
- 14 Maizels, R.M., de Savigny, D. and Ogilvie, B.M. (1984) Characterization of surface and excretory–secretory antigens of *Toxocara canis* infective larvae. *Parasite Immunol.* 6, 23–37.
- 15 Heussen, C. and Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerised substrates. *Anal. Biochem.* 102, 196–202.
- 16 Hotez, P.J. and Cerami, A. (1983) Secretion of a proteolytic anticoagulant by *Ancylostoma* hookworms. *J. Exp. Med.* 157, 1594–1603.
- 17 Hotez, P.J., Trang, N.L., McKerrow, J.H. and Cerami, A. (1985) Isolation and characterization of a proteolytic enzyme from the adult hookworm *Ancylostoma caninum*. *J. Biol. Chem.* 260, 7343–7348.
- 18 McKerrow, J.H., Pino-Heiss, S., Lindquist, R. and Werb, Z. (1985) Purification and characterization of an elastinolytic proteinase secreted by cercariae of *Schistosoma mansoni*. *J. Biol. Chem.* 260, 3703–3707.
- 19 Raybourne, R., Deardorff, T.L. and Bier, J.W. (1986) *Anisakis simplex*: Larval secretory protein production and cytostatic action in mammalian cell cultures. *Exp. Parasitol.* 62, 92–97.
- 20 Kennedy, M.W., Gordon, A.S., Tomlinson, L.A. and Qureshi, F. (1987) Genetic (Major Histocompatibility Complex?) control of the antibody repertoire to the secreted antigens of *Ascaris*. *Parasite Immunol.* 9, 269–273.
- 21 Smith, J.W. (1983) *Anisakis simplex* (Rudolphi, 1809, det Krabbe, 1878) (Nematoda: Ascaridoidea): morphology and morphometry of larvae from euphausiids and fish, and a review of the life-history and ecology. *J. Helminthol.* 57, 205–224.
- 22 Qureshi, F., Kennedy, M.W., Gibson, S., Ye, P.Z. and Miller, H.R.P. (1987) Resistance of nematode secretory products to cleavage by mast cell proteinases. *Mol. Biochem. Parasitol.* 24, 137–145.
- 23 Meghji, M. and Maizels, R.M. (1986) Biochemical properties of larval excretory–secretory glycoproteins of the parasitic nematode *Toxocara canis*. *Mol. Biochem. Parasitol.* 18, 155–170.
- 24 Maizels, R.M., Philipp, M. and Ogilvie, B.M. (1982) Molecules on the surface of parasitic nematodes as probes of the immune response in infection. *Immunol. Rev.* 61, 109–136.
- 25 Ogilvie, B.M. and de Savigny, D. (1982) Immune response to nematodes. In: *Immunology of Parasitic Infections* (Cohen, S. and Warren, K.S., eds.), pp. 715–757, Blackwell Scientific Publications, Oxford.
- 26 Jungery, M. and Ogilvie, B.M. (1982) Antibody responses to stage-specific *Trichinella spiralis* surface antigens in strong and weak responder strains. *J. Immunol.* 129, 839–843.
- 27 Tsuji, M., Hayashi, T., Yamamoto, S., Sakata, Y. and Toshida, T. (1977) IgE-type antibodies to *Ascaris* in man. *Int. Arch. Allergy Appl. Immunol.* 55, 78–81.
- 28 O'Donnell, I.J. and Mitchell, G.F. (1980) An investigation of the antigens of *Ascaris lumbricoides* using a radioimmunoassay and sera of naturally infected humans. *Int. Arch. Allergy Appl. Immunol.* 61, 213–219.
- 29 Robertson, B.D., Rathaur, S., McKerrow, J.H. and Maizels, R.M. (1987) Functional analysis of the excretory–secretory molecules of *Toxocara canis* infective larvae. *J. Cell. Biochem. Suppl.* 11A, 328.

- 30 Knox, D.P. and Kennedy, M.W. (1988) Proteinases released by the parasitic larval stages of *Ascaris suum*, and their inhibition by antibody. *Mol. Biochem. Parasitol.* 28, 207–216.