Non-covalent interactions result in aggregation of surface antigens of the parasitic nematode *Trichinella spiralis*

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Surface antigens of three stages of the nematode worm *Trichinella spiralis* have been labelled with iodine and examined by sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions. A variety of interactions were defined: the infective larva surface antigens formed a spectrum of aggregates from 50 kDa to >1000 kDa from subunits of 47 kDa and 90 kDa; in the adult worms a 60 kDa complex arose from interaction between two dissimilar molecules of 40 kDa and 20 kDa; the new-born larvae components formed homologous dimers from a 58 kDa molecule. Aggregating molecules were adherent to lentil lectin–Sepharose and are therefore glycoproteins. The interactions observed were completely abolished by boiling in SDS/mercaptoethanol, but only partially destroyed by boiling in SDS/iodoacetamide. Based upon this, the associations can be characterized as non-covalent, but disulphide-bond-dependent. It is suggested, but not proved, that the aggregates arise from strong non-covalent hydrophobic interaction sites which are stabilized by intrachain disulphide bonds in the molecules concerned.

About three-quarters of the world population are infected with one or other parasitic worm (Wood & Rue, 1980). Many of these worms belong to the phylum Nematoda, which include amongst their members not only parasitic but also free-living forms.

All nematodes are surrounded by a multilayered flexible exoskeleton, the cuticle, which is shed and replaced four times by larval forms before they reach the reproductive adult stage. These four moults define the characteristic five-stage life cycle of nematodes.

The organization of the cuticle at the ultra-structural level has been well described (Bird, 1971, 1980; Lee, 1966, 1972) but information about its biochemical composition is scarce. Moreover, at the molecular level the organization of the cuticle is largely unknown, although collagen-like molecules are thought to play an important part (Bird, 1971, 1980; Lee, 1966, 1972).

In recent studies, the parasitic nematode *Trichinella spiralis* has been surface-labelled with iodine (Philipp *et al.*, 1980; Parkhouse *et al.*, 1981). The radiolabelled products were shown to be very

few in number (two to four components), antigenic in infected hosts and stage-specific (Philipp *et al.*, 1981; Parkhouse *et al.*, 1981). In addition intestinal worms and infective larvae cultured *in vitro* were shown to shed radiolabelled surface antigens at a rate depending on the culture conditions (Philipp *et al.*, 1980).

In this paper we extend the biochemical characterization of the surface antigens of *T. spiralis* and show that most of them are glycoproteins and that many of these form non-covalent aggregates. These aggregates are strongly disulphide-bond-dependent in that dissociation is greatly facilitated when intramolecular disulphide bonds are chemically reduced.

Materials and methods

Maintenance of T. spiralis

The *T. spiralis* life cycle was maintained in outbred Parke's mice or August rats. Intestinal worms, new-born larvae and infective larvae were recovered as described by Dennis *et al.* (1970).

Surface labelling of worms

Carrier-free radioactive iodide (50–500 μ Ci; The Radiochemical Centre, Amersham, U.K.) was dis-

Abbreviation used: SDS, sodium dodecyl sulphate.

pensed into an acid-washed glass test tube and about 50 μ l of packed worms resuspended in 70–100 μ l of phosphate-buffered saline (130 mM-NaCl/4 mM-KCl/8.1 mM- Na_2HPO_4 /1.9 mM- NaH_2PO_4 , pH 7.4) was added. Radio-iodination of tyrosine residues was then effected by adding at room temperature chloramine T (0.8 mg/ml in water) in the form of two additions of 5 μ l separated by 2 min. After a further 2 min at room temperature, the worms were transferred to a 10 ml conical plastic centrifuge tube, and washed by sedimentation until no further radioactivity appeared in the supernatant. On some occasions a drop of aqueous saturated tyrosine was added to the worms at the end of the labelling period. This simply consumes unreacted iodine, but does not change the result of labelling. This procedure selectively labels the surface (Philipp *et al.*, 1981; Parkhouse *et al.*, 1981).

Solubilization of the labelled worms

The washed, labelled worms were resuspended in 0.5 ml of 10 mM-Tris adjusted to pH 8.0 by addition of HCl (Tris buffer) containing 50 μ g of 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK') and 25 μ g of 7-amino-1-chloro-3-L-tosyl-amidoheptan-2-one ('TLCK')/ml. These were included as inhibitors of proteolysis. As a further proteinase inhibitor, 100 mM-phenylmethanesulphonyl fluoride (Sigma) in isopropanol (5 μ l) was added to the worms in suspension, which were then disrupted by using a glass homogenizer (infective larvae and adults) or by sonication (new-born larvae). Then 10% (w/v) sodium deoxycholate in 10 mM-Tris/HCl buffer, pH 8.0 (120 μ l) was added, and the mixture was further homogenized. All of the solubilization steps were conducted at a temperature of 0°C. After standing the homogenate on ice for 10–20 min, it was centrifuged at 4°C and 25 000 *g* for 30 min.

At least 90% of the radioactivity bound by infective larvae and adult worms was solubilized by this procedure, whereas approx. 50% of the radioactivity incorporated by the new-born larvae was solubilized. The insoluble material was discarded.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was carried out on 7.5% (w/v) polyacrylamide gel slabs using the Pharmacia equipment (GS C-8) and the buffer gel systems suggested by the manufacturer in their instruction leaflet. As a molecular weight marker the Pharmacia kit containing phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soya bean trypsin inhibitor (M_r 20 000) and α -lactalbumin (M_r 14 400) was labelled with ^{125}I Bolton–Hunter reagent as suggested by the suppliers (The Radio-

chemical Centre, Amersham, U.K.), and at a specific activity of 20–100 μCi per 50 μg of total protein. Following electrophoresis, the gel slabs were fixed in acetic acid/methanol/water (3:10:27, by vol.), dried down and autoradiographed at -70°C using an Ilford intensifying screen and hypersensitized film (Laskey & Mills, 1977). The film was processed according to the manufacturer's instructions. Samples for electrophoresis were first heated for 3 min at 100°C under reducing or non-reducing conditions. For effective reduction the sample was heated in an aqueous solution containing SDS (16.7 mg/ml), glycerol (8.3 mg/ml), Trizma base (Sigma) (0.7 mg/ml), disodium EDTA (3.7 mg/ml), Bromophenol Blue (1.7 μg /ml) and 2-mercaptoethanol (8.3 mg/ml). Samples to be processed under non-reducing conditions were treated in a similar manner except that iodoacetamide (16 mg/ml) replaced the 2-mercaptoethanol, and the solution containing the iodoacetamide was always prepared on the day of use.

SDS/polyacrylamide rod gel electrophoresis was carried out in 8 cm gel columns in glass tubes (0.6 cm internal diameter) on 5% (w/v) acrylamide according to published specifications (Summers *et al.*, 1965).

Lectin affinity chromatography

Lentil lectin–Sepharose was obtained from Vector Laboratories, Burlingame, CA, U.S.A., and

Fig. 1. Surface antigens of three stages of *T. spiralis* Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Infective larvae (*a*, *b*) newborn larvae (*c*, *d*) and 6-day-old adults (*e*, *f*) were labelled with ^{125}I , solubilized and electrophoresed on gel slabs under non-reducing (*a*, *c*, *e*) or reducing (*b*, *d*, *f*) conditions. Details are given in the Materials and methods section. The position of the molecular weight markers run in parallel is indicated on the figure.

stored in 0.15 M NaCl/0.5 mM-CaCl₂/0.5 mM-MnCl₂/0.1% NaN₃/50 mM-Tris/HCl buffer, pH 7.5.

Prior to use columns of lectin-Sepharose were washed with 10 mM-Tris/HCl buffer, pH 8.0, followed by 0.5% sodium deoxycholate/10 mM-Tris/HCl buffer, pH 8.0. The deoxycholate solubilized worm extract was then applied and after extensive washing the bound fraction was eluted with 0.2 M- α -methyl mannoside (or glucose) in 0.5% sodium deoxycholate/10 mM-Tris/HCl buffer, pH 8.0. Both adherent and non-adherent fractions were analysed by SDS/polyacrylamide-gel electrophoresis without concentration or further manipulation.

Results

Infective larvae, adult worms and new-born larvae were labelled with iodine, solubilized and submitted

to polyacrylamide-gel analysis under reducing and non-reducing conditions (Fig. 1). Under reducing conditions the labelled components were resolved thus: infective larvae, 105, 90, 55 and 47 kDa (Fig. 1b); new-born larvae, 64, 58, 34 and 30 kDa (Fig. 1d); adult worms, 40, 33 and 20 kDa (Fig. 1f). The pattern was quite different on the non-reducing gels. Infective larvae yielded a continuum of molecular size from approx. 100 kDa to >1000 kDa (Fig. 1a) in addition to the 55 and 105 kDa bands. New-born larvae were resolved into four components of 120, 60, 34 and 32 kDa (Fig. 1c), and adults had mainly molecules of 60 and 30 kDa (Fig. 1e).

At first sight, the simplest conclusion would be that assembly of surface antigens into multi-chain molecules occurs by disulphide-bond linkages. This is not, however, so. Instead, as will be indicated below, molecular aggregates relatively resistant to

Fig. 2. Disulphide-bond-dependent aggregate formation by surface antigens of infective larvae of *T. spiralis*. Infective larvae were labelled with ¹²⁵I, solubilized and electrophoresed in a 5% (w/v) gel cylinder. The gel was sliced with a razor blade assembly, counted and the results are plotted in the top of the figure. Slices labelled a–f were extracted and re-electrophoresed on gel slabs under non-reducing (middle of the figure) or reducing conditions (bottom of the figure) as indicated. The two gels labelled t represent analysis of total labelled worms without the first fractionation step on the disc gel. The horizontal lines in the middle and bottom of the figure are included for orientation and indicate the position of the 90 kDa component. Details are in the Materials and methods section.

dissociation by sodium dodecyl sulphate are formed in a disulphide-bond-dependent, rather than disulphide-bond-linked, manner.

The experimental design was first to separate iodine-labelled antigens by sodium dodecyl sulphate/polyacrylamide-gel analysis on a rod gel under non-reducing conditions. Then, having located the various components by slicing and counting, individual radioactive antigens were extracted and then re-analysed on gels under reducing and non-reducing conditions.

In Fig. 2 is presented a composite of such an experiment done with the infective larvae. As would be expected (Fig. 1*a*), a complex pattern results from electrophoresis under non-reducing conditions (Fig. 2, top and track *t*). When selected fractions *a*–*d* in Fig. 2 were re-run several conclusions emerged. On reducing gels (Fig. 2, bottom) all of the higher molecular weight material was now resolved into the two components of 90 and 47 kDa. The very same samples re-run on non-reducing gels, however, did not re-run true. Instead they yielded the same two 90 and 47 kDa components as well as a range of larger aggregates. For example, fraction *a*, which should have re-run entirely at the top of the non-reducing gel (Fig. 2, middle), yielded a range of components, and the same result was obtained when the high-molecular-weight aggregate at the top of the re-run gel was extracted once again and re-run a further time on SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (results not shown). These findings thus indicate that the 90 and 47 kDa components which, as will be demonstrated below, are glycoproteins, form high-molecular-weight aggregates partially resistant to the dissociating action of SDS under non-reducing conditions, but dissociable under reducing conditions. This is not the case with the 105 and 55 kDa components. As can be seen, both re-electrophorese true when examined on a non-reducing gel (Fig. 2, fractions *e* and *f*). There is, however, evidence for the formation of dimers by the 55 kDa component (compare the reduced and non-reduced gels of fraction *e*, Fig. 2). However, if the SDS/polyacrylamide-gel electrophoresis run and re-run experiment is performed under reducing conditions for both steps, all four components (105, 90, 55 and 47 kDa), re-run true (results not shown).

Similarly designed experiments demonstrate the existence of SDS-resistant disulphide-bond-dependent aggregates in surface antigens of adults and newborn larvae. For new-born larvae, the labelled antigens of approx. 120 and 60 kDa were recovered from a non-reducing gel and then re-examined under reducing and non-reducing conditions (Fig. 3). As can be seen, the approx. 120 kDa molecule appears as a 58 kDa band when re-run on reducing gels, but is localized both in the 56 and 120 kDa part of a

Fig. 3. Disulphide bond aggregate formation by surface antigens of adult and new-born larvae of *T. spiralis*. Worms were labelled, electrophoresed under non-reducing conditions and selected bands were re-run reduced and non-reduced as indicated in the legend to Fig. 2. From the sample of newborn larvae, the bands at 120 (*a*, *b*) and 60 (*c*, *d*) kDa were re-run unreduced (U) and reduced (R). From the sample of the adult, the band running at about 60 kDa (*e*, *f*) was rerun unreduced (U) and reduced (R). The position of the molecular weight markers run in parallel is indicated on the Figure.

non-reducing gel (Fig. 3*a*). The 58 kDa band must therefore form dimers to yield the approx. 120 kDa band. The 60 kDa component re-runs true on non-reducing gels, but is slightly slower (apparently 64 kDa) on reducing gels (Fig. 3*d*). This type of apparent molecular weight change is commonly observed when restrictions imposed by intrachain disulphide bonds are relieved by chemical reduction. The adult 60 kDa antigen recovered from non-reducing gels totally dissociates into 40 and 20 kDa components when re-electrophoresed in reducing gels; under non-reducing conditions this dissociation is partial (Fig. 3*e*). Thus both the adult and new-born larvae surface antigens include components consisting of aggregates which partially dissociate in SDS. Complete dissociation results only when mercaptoethanol is included in the SDS.

Detergent extracts of radiolabelled worms were passed over columns of lentil lectin–Sepharose in order to determine which components are glycoproteins (Fig. 4). By this criteria all antigens described above (Fig. 1) were glycoproteins, with the exception of the 150 and 55 kDa components of the infective larvae and the two closely opposed bands, approx. 32 kDa, of the new-born larvae. The additional adult glycoprotein antigen of 56 kDa not

Fig. 4. *Lentil-lectin-binding surface antigens of T. spiralis*

Radioactive labelled lysates of infective larvae (a, b), 2-day-old intestinal worms (c, d) and new-born larvae (e, f) were passed through columns of lentil lectin-Sepharose and the bound fraction was eluted with 0.2% α -methylmannoside. The total unfractionated material (a, c, e) and that bound to and eluted from lentil lectin-Sepharose (b, d, f) were analysed on gels under reducing conditions except for those from newborn larvae (e, f) which were run under non-reducing conditions. Details are in the Materials and methods section. The position of the molecular weight markers run in parallel is indicated on the Figure.

seen in Fig. 1 is present on 1–2-day-old intestinal worm populations but absent from later more mature forms.

Discussion

The parasite surface constitutes an essential site for recognition and elimination by the immune system. Unravelling the organization of the cuticle at the molecular level, as well as its antigenic nature, is therefore a necessary first step in the constitution of a programme aimed at the understanding and eventual destruction of parasitic organisms.

The main finding to emerge from these studies is that surface antigens of each stage of *T. spiralis* form multi-chain molecular aggregates in a disulphide-bond-dependent manner. How such aggregates are organized in the surface of the living worm

is a matter of conjecture, but it does seem reasonable to suggest that this common structural feature must be important in the organization of the worm's surface.

The evidence for the existence of aggregate formation is simply the comparison between the patterns obtained on SDS gels when the samples are electrophoresed reduced and not reduced. Thus the infective larvae antigens are composed of two molecules (105 and 55 kDa) that do not adhere to lentil lectin and which do not tend to form large aggregates, and two glycoproteins (90 and 47 kDa) which do. In the adult a 60 kDa complex is formed by the union of two glycoprotein molecules (40 and 20 kDa), and in the new-born larvae, a 58 kDa glycoprotein antigen dimerizes. The molecular interaction producing all of these aggregates is completely destroyed by boiling in SDS/mercapto-

ethanol and partially affected by boiling in SDS/iodoacetamide. The partial dissociation under such non-reducing conditions suggests that the interaction is non-covalent, but is certainly largely resistant to dissociation by SDS. It is, however, disulphide-bond-dependent, since complete dissociation takes place in SDS/mercaptoethanol. This deduction does depend upon the unproven assumption that there have been no uncontrolled oxidative processes, but it should be stated that identical procedures never cause dissociation of disulphide-linked immunoglobulin subunits (Parkhouse & Askonas, 1969). With this in mind, the most rational explanation is that strong hydrophobic interaction sites mediate the associations observed, and that their integrity is stabilized by intrachain disulphide bridges in the molecules concerned. In the specific context of nematode surfaces, however, such non-covalent interactions may help to explain the phenomenon of shedding of nematode surface antigens (Philipp *et al.*, 1980).

Finally, the occurrence of SDS-resistant, non-covalent, hydrophobic associations in surface structures may be a general phenomenon, since they have also been noted in the HLA-DR antigens of human lymphoid cells (Springer *et al.*, 1977), and the red cell transmembrane component glycoprotein (Furthmayr & Marchesi, 1976).

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