Extracellular and cytoplasmic Cu/Zn superoxide dismutases from *Haemonchus contortus*

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SUMMARY

Full-length cDNAs encoding cytosolic (SODc) and putative extracelluar (SODe) Cu/Zn superoxide dismutases (SODs) from the ovine gastrointestinal parasitic nematode *Haemonchus contortus* have been isolated and characterized. The predicted sequences of the *H. contortus* SODs showed strong homology to other helminth SODs, the highest level of sequence similarity was with those of the free-living nematode *Caenorhabditis elegans*‡. The predicted amino acid sequence of the putative extracellular form contained an N-terminal extension with the characteristics of a signal sequence including a potential signal peptidase cleavage site. Transcripts of both classes of Cu/Zn SOD were detected in all life-cycle stages examined. The cytosolic SOD mRNA was approximately 6-fold more abundant than that of the extracellular enzyme in adult parasites. Immunoblotting with antisera raised to *in vitro*-expressed parasite SODs revealed the presence of 2 proteins in extracts of adult *H. contortus*, with molecular masses of approximately 19·8 and 18 kDa. An additional protein of approximately 16·8 kDa was detected in adult ES material. Immunofluorescent staining showed Cu/Zn SOD was localized in the body wall musculature and the pharynx in adult worms and in the uterine tract of adult females. The immunogenic properties of recombinant *H. contortus* Cu/Zn SODs was assessed in a challenge infection experiment in lambs.

Key words: *Haemonchus contortus*, superoxide dismutase, extracellular, RT–PCR, immunogenicity.

INTRODUCTION

The sustained release of toxic free-radicals, such as the superoxide ion (O_2^-), by activated phagocytes may play a role in the killing of parasites. The parasite may counteract these responses using anti-oxidant enzymes, such as superoxide dismutase (SOD) (Callahan, Crouch & James, 1988). SODs catalyse the dismutation of O_2^- to hydrogen peroxide and ground-state oxygen, and consequently are considered to protect cells from this damaging by-product of oxygen metabolism (Fridovich, 1995). SODs have been classified into 3 groups based on the metal ion contained in the active site: Mn, Fe and Cu/Zn. The Mn and Fe SODs are structurally related to one another, but differ from the Cu/Zn SODs. Recently, the Cu/Zn-dependent SODs have been subdivided into 2 types, cytosolic and extracellular enzymes (Fridovich, 1995). Both forms of Cu/Zn SODs have been reported in parasitic helminths (James, 1994).

There is increasing evidence that anti-oxidant enzymes facilitate the survival of helminths in the gastrointestinal tract. Elevated levels of key anti-oxidant enzymes, such as SOD, catalase and glutathione peroxidase have been correlated with *N. brasiliensis* persistence in the intestine of its rodent host (Smith & Bryant, 1986; Batra *et al.* 1993). SOD enzyme activity and isoenzyme polymorphism were shown to be elevated in 'adapted' *N. brasiliensis* capable of surviving in primed hosts, compared to worms harvested after a primary infection of naive rats (Knox & Jones, 1992). Increased free-radical production by peritoneal leukocytes has been correlated with the rejection of *N. brasiliensis* and *Fasciola hepatica* (Smith & Bryant, 1989*a*; Smith, Ovington & Boray, 1992). Finally, *N. brasiliensis* rejection can be inhibited by the administration of anti-oxidants (Smith & Bryant, 1989*b*).

In ruminants, the contribution of free-radicals to the expulsion of gastrointestinal nematodes has been less well studied. Expulsion of the ovine gastrointestinal nematodes *H. contortus* and *Trichostrongylus vitrinus* was accelerated when molybdenum was added to the host diet; it was suggested that the molybdenum may have enhanced the inflammatory reaction in the intestinal mucosa (Suttle *et al.* 1992*a, b*). Sera from cattle immunized against the bovine lungworm *Dictyocaulus viviparus* recognized and neutralized the activity of the parasite excreted/secreted SOD (Britton, Knox & Kennedy, 1994). Several anti-protozoal agents (Docampo & Moreno, 1986; Smith, Bryant & Boreham, 1988) and some anthelmintic agents such as ascaridol, an endo-

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peroxide, and the salicylanilides may act as free-radical generators (Bennet-Jenkins & Bryant, 1996). Endoperoxides can kill *H. contortus* 3rd-stage larvae (L3) *in vitro* and can reduce faecal egg output *in vivo* (Bennet-Jenkins & Bryant, 1996). Taken together, these experiments suggest that free-radical generation is an important effector mechanism for worm expulsion. Thus, parasite free-radical scavenging enzymes such as SOD may be crucial for parasite survival and may be appropriate targets for the development of novel immune or chemical-based control procedures.

The study described here extends the findings that several distinct isoforms of Cu/Zn SODs are present in *H. contortus* (Knox & Jones, 1992) and that SOD activity can be detected in the culture medium following the *in vitro* culture of adult parasites (Knox, D. P., unpublished observations). The dynamics of SOD expression and the localization of the enzyme in *H. contortus* were examined, and the effect of systemic vaccination with recombinant parasite SOD on the outcome of infection was investigated.

MATERIALS AND METHODS

Parasites

Third-stage larvae (L3) were cultured from faeces of previously worm-free donor lambs which were experimentally infected with *H. contortus*. L3 were exsheathed (XL3) by exposure to 0·2% sodium hypochlorite (E. Jackson, personal communication). Fourth-stage larvae (L4), immature (11 day) and mature adults (22 and 28 day) were harvested from the abomasa of donor lambs infected with 20–50 000 L3, as described previously (Smith & Smith, 1993). Parasites were stored in liquid nitrogen until required.

Isolation of Cu/Zn SOD encoding cDNAs by PCR

Double-stranded cDNA was prepared as previously described (Redmond *et al*. 1997). Initial amplifications were performed using the nematode spliced leader sequence, SL1 (Krause & Hirsch, 1987) and a degenerate antisense primer 5′-ACCGCA-IGCGAGICGAGCICCAGCITTACCAGT-3′ {I = inosine} designed to the highly conserved region surrounding the active site residue arginine (arginine-139 in *C. elegans* E, Fig. 1). PCR cycles used were: 94 °C for 45 s, 30–60 °C (depending on the primers used) for 1 min, 72 °C for 2 min, for 30 cycles, followed by an 8 min incubation at 72 °C. Full length cDNAs for each class of enzyme were isolated using 5′ gene-specific primers and an oligo-d(T) primer. Amplified products were subcloned into the pCRII vector (Invitrogen) and their DNA sequences were determined using the T7 Sequencing Kit™ (Pharmacia). The UWGCG sequence analysis software package, version 8.0–UNIX, 1994 was used in nucleic acid and amino acid sequences. Potential signal peptide cleavage sites were identified by the method of Nielsen *et al*. (1997). Phylogenetic analysis was performed using the Pileup, Distances and Growtrees programs in the GCG package.

In vitro *expression and purification of* H. contortus *Cu/Zn SODs*

The entire coding region of SODc and the postulated mature SODe coding region were ligated into pET22b(+) (Novagen), following the incorporation, by PCR, of an *Nde*I site at the start codon and an *Eco*RI site following the termination codon. A new methionine initiation codon was engineered immediately before the first codon of the putative mature protein sequence of SODe. Primers used were SODc-ET5′(5′-AGTCGGATCCATATGA-GTAACCGTGCTGTTG-3′), SODc-ET3′(5′-G-TCTAGAATTCAGTCACTGGGGAGCAGC-3′), SODe-ET5′(5′-AGTCGGATCCATATGTA-TATCTTTGAAGCAGTG-3′), SODe-ET3′(5′-GTCGCGAATTCCGTTTAGAGGATACCG-3′). Resultant constructs were expressed in *Escherichia coli* BL21 cells (procedure as pET System Manual, Novagen). Cells were harvested by centrifugation (11 000 ***g***) 5 h after induction of recombinant protein expression, washed by resuspension in ice-cold PBS, then resuspended and incubated in 0·75 M sucrose, 10 mM Tris–acetate (pH 7·5), 1·5 mM EDTA, 100 μg/ml lysozyme, 10 μg/ml DNase and RNase, 8 mM $MgCl_2$ on ice, for 30 min. Soluble and insoluble fractions were separated by centrifugation (11 000 ***g***). Recombinant SODc with an estimated molecular mass of 18 kDa, was purified from the soluble fraction using DEAE-cellulose chromatography. HPLC was performed on a TSK DEAE SPW column (5 mm × 75 mm; Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7·5). Recombinant SODc was found in the unbound material, separated from the majority of the contaminating bacterial cellular proteins. This material was dialysed to 50 mM Tris–HCl (pH 8·9) and reapplied to the column which had been equilibrated in the same buffer. Recombinant SODc was again found in the unbound fraction. Recombinant SODe in *E. coli* inclusion bodies was solubilized in 10 M urea/50 mM Tris–HCl, pH 7·5 (on ice, 2 h), the solution was clarified by centrifugation and the urea removed by dialysis. Purified, soluble, recombinant SODe was obtained by a similar method to that for SODc using 1 ml of DE–52 (pH 7·5) in low pressure chromatography columns (5 mm × 50 mm), rather than HPLC. The recombinant SODs were analysed using 15% SDS–PAGE according to standard protocols (Laemmli, 1977; Sambrook, Fritsch & Maniatis, 1989) and enzymatic activity was examined by

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fractionation on polyacrylamide gels followed by visualization of zones of activity by the photochemical reduction of nitroblue tetrazolium (Beauchamp & Fridovich, 1971).

Generation of polyclonal antisera against H. contortus *Cu/Zn SODs and Western blotting*

Polyclonal antisera were raised in rabbits by intramuscular inoculation of soluble recombinant protein (25 μg SODe or 50 μg SODc) emulsified in Freund's Complete Adjuvant, followed by 2 booster injections of the recombinant protein in Freund's Incomplete Adjuvant at 4 week intervals. Rabbits were bled 14 days after the final booster injections and the sera designated anti-HCSODc and anti-HCSODe.

Protein extractions from adult *H. contortus* (28 days) were prepared as described previously (Smith, Smith & Murray, 1994) where the first supernatant is denoted S1 (cytosolic material), the second S2 (carry-over cytosolic material and loosely membrane associated components), and the final suspension S3 (membrane-bound material). ES material was prepared from adult parasites as previously described (Young, McKeand & Knox, 1995). Samples (10 μg protein for S fractions and 15 μg for ES proteins) were fractionated by SDS–PAGE on 15% polyacrylamide gels under reducing conditions (Laemmli, 1977) and semi-dry electroblotted onto Immobilon P membrane (Millipore). Sera were depleted of *E. coli*-reacting antibodies by pre-adsorption, at the working dilution, against lysed and whole IPTG-induced *E. coli* cells containing the expression vector pET22b(+) only. Filters were probed with anti-HCSOD sera at dilutions of 1:1000. Secondary antibody was sheep anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Scottish Antibody Production Unit) at a dilution of 1:1000. Immunoreactive proteins were detected with phosphatase substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate).

Immunolocalization

Sections of adult *H. contortus* were cut and probed as described previously (Smith & Smith, 1993). Anti-HCSODc and anti-HCSODe sera were used at dilutions of 1:200 to 1:1000 (pre-adsorbed as described above). Anti-rabbit IgG fluorescein isothiocyanate (Sigma) was used as a secondary antibody conjugate at 1:100 dilution. Pre-immunization rabbit sera were used as negative controls.

Northern blotting

Total RNA was extracted as described previously (Redmond *et al*. 1997) whilst Northern blotting, hybridizations and detection of transcripts were performed according to standard techniques (Sambrook *et al*. 1989; Amersham Hybond N+ protocols). 5′ SODc- or SODe-specific fragments (C-5 and E-5) were generated by PCR from the corresponding cDNAs. C-5 was a 349 bp fragment (nts 26–375); E-5 was a 234 bp fragment (nts 20–254). Probe fragments were labelled using T7 Quickprime kit (Pharmacia). After hybridization, the filters were washed to a final stringency of 0·1×SSC, 0·5% SDS, at 42 °C.

Semi-quantitative RT–PCR

Total RNA was isolated from various parasite stages using standard methods (Chomczynski & Sacchi, 1987). Single-stranded cDNA was synthesized using the Superscript II reverse transcriptase (Gibco BRL) with the supplied oligo-dT primer. Primers were designed to distinguish cDNA products from contaminating genomic DNA products. The SODc primers generated a cDNA product of 500 nts; the SODe product was 237 nts. The *H. contortus* myosin regulatory light chain sequence (mlc) was used in parallel PCRs as the control transcript. Primers to amplify *H. contortus* mlc cDNA were designed to 2 conserved regions of the mlc sequences of *C. elegans* (Cummins & Anderson, 1988). The primers MYO-1(sense) = 5′-GGATCIGAGGCCGCCAATTCG-3′ and MYO-4 (anti-sense) = 5′-GCTCCIGT-IGTCAATIAGGTGAGC-3′ (I = inosine). The *H. contortus* cDNA product was 446 nts. Amplified fragments were separated on 0·8% agarose gels and blotted onto Hybond N+ nylon membrane (Amersham). Blots were probed with DIG-labelled mlc, SODc- and SODe-specific probes (C-5, E-5, described above). Hybridization and chemiluminescent detection of DIG-labelled probes was performed as described in the manufacturer's protocols (Boehringer Mannheim). Autoradiographs were scanned and the images quantified using ImageQuant software (Molecular Dynamics). The relative abundance of the SOD transcripts in each life-cycle were expressed as a ratio of the SOD signal to that of myosin. mRNA loading from the different parasite stages was equilibrated on the basis of OD_{260}/OD_{280} and confirmed by ethidium bromide staining.

Protection trial

Fourteen worm-free Suffolk cross Dorset lambs aged 6 months at the start of the experiment were allocated into 2 groups of 7 each balanced on the basis of sex and weight. Seven lambs were immunized 3 times intramuscularly at 3 week intervals with 100 μg recombinant SODc/SODe mixed 1/1 and emulsified in an equal volume of Freund's complete adjuvant. Control injections (7 lambs) were prepared in the same way except with PBS replacing the

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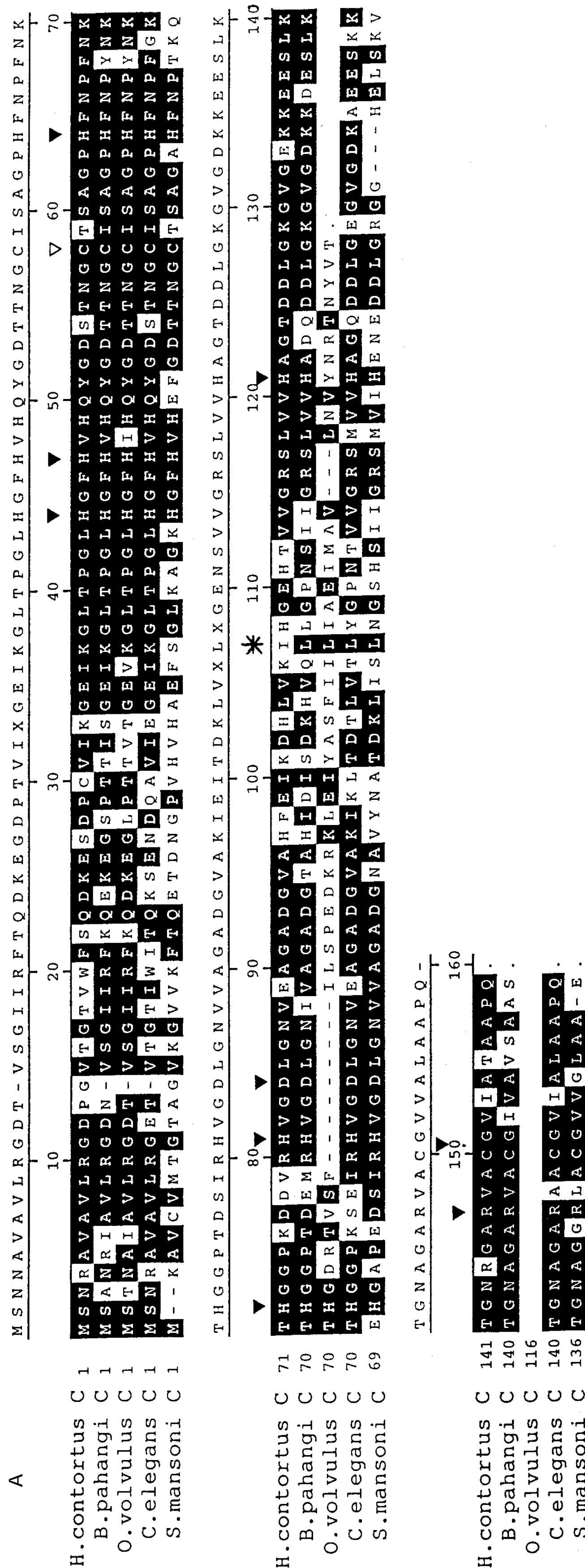

Fig. 1. For legend see facing page.

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B

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                  MXX----IFLSXIIFIXYA--V---XXHVY------N-MXXXXARX--K---AV--AVLXGDAG-VXGII
H.contortus E   1 MTM------LQQILLIS----VIIGTVHVH---------EVDCANEVLKARAYIFEAVKGGNPAKTVGII
B.pahangi E     1 MMIASFAIFLSHIIFITYAT-----SNQRYFKPNMHNNMTITIRRTITKTATAI--AVLHSDNGNINGTI
O.volvulus E    1 MINSFIVIFLSFLIFINYANLVCVEATHVYGRRSHSNGMHGNGARR------AV--AVLRGDAG-VSGII
S.mansoni E     1 M-----------------------------------------------K------AV--CVMTGTAG-VKGVV

                  XFQQXXXGXXVTISGSVSGLTPGLHGFHVHEYGDTTNGCTSAGAHFNPXKKTHGAPEDSIRHVGDLGNIV
H.contortus E  52 DLVQT--GTLVKMNGSVSGLQPGLHGFHIHEKGDLGNGCLAAGAHFNPHKMMHGAPEDSNRHVGDLGNIE
B.pahangi E   64 HFQQDKNS--TTISGEIKGLTPGLHGFHVHQYGDTTNGCISAGPHFNPYNKTHGDPTDEMRHVGDLGNIV
O.volvulus E  62 YFQQGSGGSITTISGSVSGLTPGLHGFHVHQYGDQTNGCTSAGDHYNPFGKTHGGPNDRIKHIGDLGNIV
S.mansoni E   18 KFTQETDNGPVHVHAEFSGLKAGKHGFHVHEFGDTTNGCTSAGAHFNPTKQEHGAPEDSIRHVGDLGNVV

                  AGADGXAXVYIXSDKXISLXGPHSVIGRSLVVHADXDDLGRGTGELSKESLKTGNAGGRVACGVIGIAA-
H.contortus E 120 TPKTGDTPILI-SDSVISLTGQHNVIGRAIVIHADMDDLGRGTSELSKT---TGNAGARVACGVIGIL.
B.pahangi E  132 AGADGTAHI-DISDKHVQLLGPNSIIGRSLVVHADQDDLGKGVGDKKDESLKTGNAGGRVACGIVAISA.
O.volvulus E 132 AGANGVAEVYINSYD-IKLRGPLSVIGHSLVVHANTDDLGQGTGNMREESLKTGNAGSRLACGVIGIAAV
S.mansoni E   88 AGADGNA-VYNATDKLISLNGSHSIIGRTMVIHENEDDLGRGGHELSKV---TGNAGGRLACGVIGLAA-

                  --
H.contortus E 184
B.pahangi E   200
O.volvulus E  201 S.
S.mansoni E   153 E.
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Fig. 1. Amino acid sequence alignment of selected Cu/Zn SODs. Residues are numbered from the predicted initiator methionine, numbering to the left relating to each specific SOD while the numbering above refers to the consensus sequence printed above each block. Identical amino acids are shaded black. (A and B) Alignments of cytosolic and extracellular SODs respectively. Residues involved in catalysis and metal ion binding (▼); cysteine residues used in intrachain disulphide bonding (▽). The asterisk marks where *Haemonchus contortus* SODc differs to the 23 invariant residues identified by Getzoff *et al*. 1989. Arrows indicate the potential signal peptide cleavage sites.

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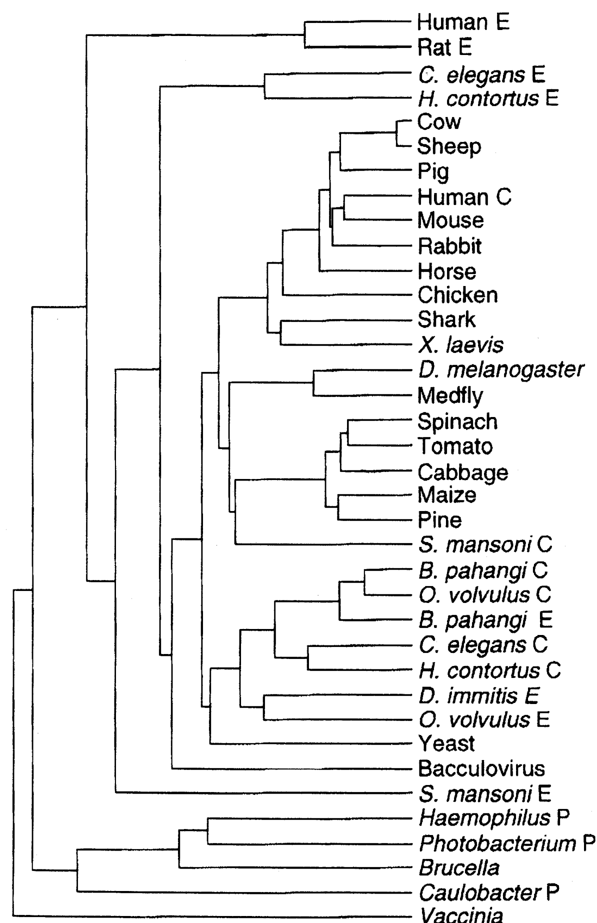

Fig. 2. A computer-generated phylogenetic tree of relationships from the predicted amino acid sequences of selected Cu/Zn SODs. Sequences used here are present in the EMBL, Genbank and Swissprot databases. All sequences are cytosolic unless marked E (extracellular) or P (periplasmic).

antigen. The preparations were injected intramuscularly into 1 site on each hind leg using 2 ml for each immunization. Lambs were challenged with 5000 *H. contortus* larvae 2 weeks after the final injection and killed 5 weeks later. Faecal egg counts were made 3 times a week from 14 days after challenge. Final worm burdens were calculated from aliquots of abomasal contents and mucosal washings.

RESULTS

Isolation of H. contortus *Cu/Zn SOD-encoding cDNAs*

Two distinct classes of Cu/Zn SOD-encoding cDNAs were isolated from *H. contortus* by PCR (*H. contortus* C and *H. contortus* E in Fig. 1A and B respectively). Analysis of the predicted protein sequences of the 2 classes of cDNA indicated that one class encoded a cytoplasmic Cu/Zn SOD (SODc, Fig. 1A), and the other encoded a putative extracellular form of the enzyme (SODe, Fig. 1B) since it contained a hydrophobic N-terminal sequence with the characteristics of a signal sequence (Dalbey & Von Heijne, 1992). Putative signal peptidase cleavage sites were identified in the predicted polypeptide sequence, immediately following alanine-25 and alanine-33 and, by analogy to existing SODe sequences from other helminths, the latter was selected as the most likely cleavage site to yield the mature enzyme after expression cloning. The predicted molecular weights of the proteins encoded were SODc, 19·343 kDA; full-length SODe, 22·339 kDa; SODe cleaved at A33, 18·116 kDa and SODe cleaved at A25, 19·231 kDa. The 2 *H. contortus* SODs shared 48% amino acid identity over their core sequences and showed strong homology to other helminth SODs, in particular their counterparts from *C. elegans*. Each SOD contained 1 potential site for N-linked glycosylation at asparagine 65 (SODe) and asparagine 69 (SODc).

Several isolates of each class of Cu/Zn SOD cDNA from independent PCR amplifications were cloned and sequenced to eliminate *Taq* DNA polymerase-induced variations in the final sequences. All of the SODc cDNA sequences isolated were identical, while 6 non-identical variants of SODe were obtained. These cDNAs differed from one another over their entire length by 0·5–3·3%. The majority of the 39 nucleotide changes found were in the 3rd position of codons or in the non-coding portion of the sequence and therefore would have no effect on the potential protein sequence. Only 4 changes would result in amino acid substitutions and these were either conservative substitutions or were in regions of the peptide sequence that are not strongly conserved. Genomic Southern blots (data not shown) indicated that SODe is encoded by multiple sequences whilst SODc is present as a single copy gene.

A phylogenetic tree constructed from the predicted Cu/Zn SOD sequences from a variety of sources (Fig. 2) showed that the *H. contortus* and *C. elegans* extracellular SODs were found on a different branch to their respective cytosolic counterparts which, in turn, were grouped with the cytosolic and extracellular SODs from filarial nematodes.

In vitro *expression of parasite SODs*

The majority of recombinant SODc produced was highly soluble, enzymically active as judged using a gel-based assay, activity being inhibited by EDTA and cyanide (data not shown), indicating a Cu/Zn-dependent enzyme. The recombinant enzyme was purified and used to raise a polyclonal antiserum. Recombinant parasite SODe was also produced in the same expression system in an identical manner. In this case, however, both the full-length enzyme and a truncated version lacking the putative hydrophobic signal peptide, were insoluble. Attempts made to generate a soluble form of recombinant

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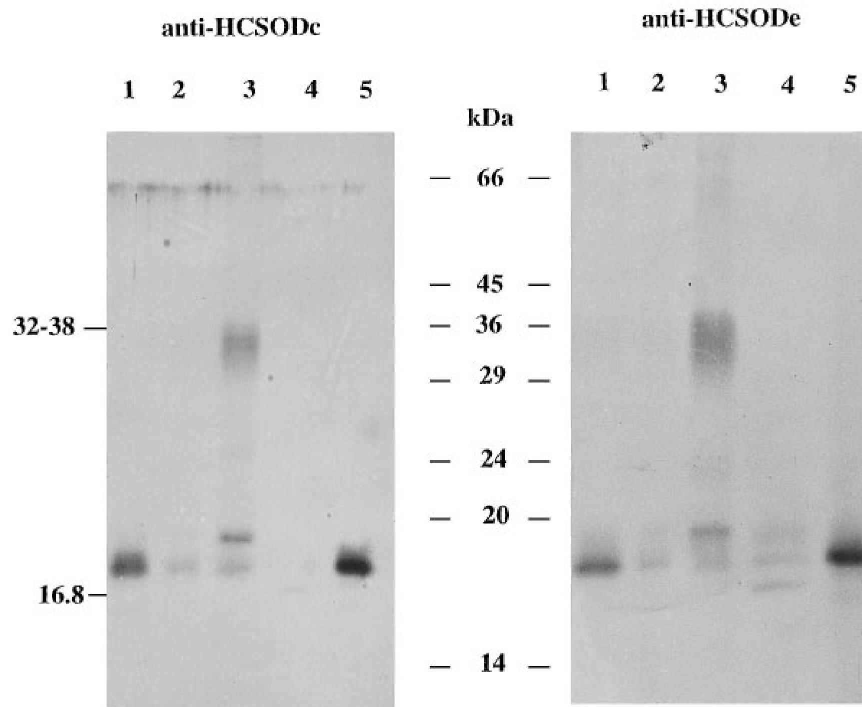

Fig. 3. Detection of Cu/Zn SODs in adult *Haemonchus contortus*. Protein samples are water soluble: Lane 1, membrane associated; Lane 2, membrane bound; Lane 3, adult ES; Lane 4, purified recombinant SODc; Lane 5, ES material was only recognized by the anti-HCSODe serum. Purified recombinant SODc is shown as the positive control, and illustrates the cross-reactivity of the anti-HCSODe serum with the cytosolic SOD. Molecular weight markers are in kiloDaltons.

SODe had limited success, although sufficient quantities of purified, soluble SODe were obtained to allow generation of a polyclonal antiserum.

Localization of Cu/Zn SODs in adult parasites

Antisera raised against recombinant SODc and SODe strongly bound both recombinant proteins with a high degree of cross-reaction evident. In adult parasite extracts the antisera bound two peptides predominantly with estimated molecular masses of 18 kDa and 19·8 kDa (Fig. 3). The 18 kDa protein was the only protein recognized in the soluble (S1) fraction and is likely to be the cytosolic form of the enzyme, having the same molecular mass as recombinant SODc. The 19·8 kDa protein was found in the membrane-associated (S2) and membrane-bound (S3) fractions, indicative of a membrane association for SOD. Anti-HCSODe clearly recognized 3 components in ES material including a 16·8 kDa component which was not evident in the other samples probed. Finally, another region of 32–37 kDa in size was recognized in the membrane-bound fraction (S3). Binding seemed to be associated with a SODe-specific component, since it was removed by pre-adsorption of the anti-SODc serum with recombinant SODe (the converse was not true).

The tissue localization of Cu/Zn SODs in adult *H. contortus* was investigated in adult worms using indirect immunofluorescent staining (Fig. 4). Essentially the same pattern of staining was observed with antisera raised against the 2 classes of Cu/Zn SOD, although the level of signal seen with the anti-HCSODc serum was considerably stronger than that obtained with the anti-HCSODe serum. SOD was not distributed evenly throughout the adult worm tissues but was localized primarily to the body wall muscle cells and lateral cords (Fig. 4A) with some staining in the luminal surface of the pharynx (Fig. 4B) and in the uterine ducts of females (Fig. 4C). The staining noted in the uterus was observed in only a fraction (30 %) of female worm sections.

Expression of H. contortus *Cu/Zn SODs during the life-cycle*

Expression of the Cu/Zn SOD genes was examined initially in *H. contortus* adult stages by Northern blot analysis. cDNA fragments specific to the region of greatest sequence diversity (in the 5′ sequence) were used to probe mRNA from adult worms. The SODe cDNA fragment probe hybridized to a single band approximately 840 nucleotides long, while the SODc probe hybridized to a single band approximately 630 nucleotides in length (Fig. 5). Densitometric scanning indicated that the SODc transcript was 6-fold more abundant than the SODe transcript in 11-day-old adults and in 28-day-old adults (not shown).

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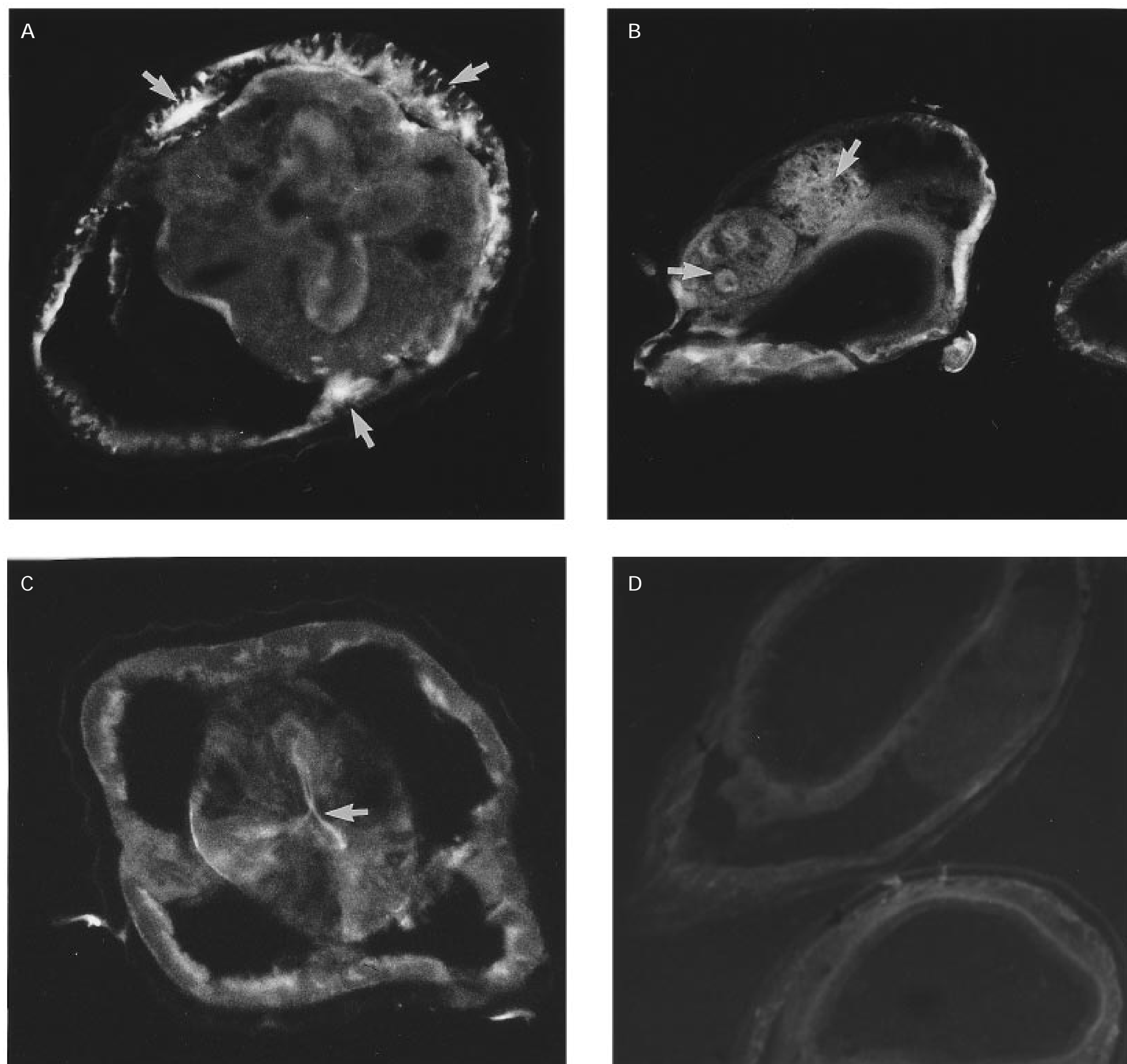

Fig. 4. Immunofluorescent staining of transverse sections of *Haemonchus contortus* adults using the anti-HCSODc serum (1:200 dilution). Strong fluorescence was evident in the body wall musculature and the lateral cords (A, arrowed), in the oesophago-pharyngeal region (B) and the uterine tract in female worms (C). Fluorescence was absent in sections probed with the pre-immunization rabbit serum (D).

The difference in the SODc and SODe transcript levels in adults seen in Northern blots was confirmed by semi-quantitative RT–PCR and was shown to be maintained in the other stages examined; the 2 classes of SOD transcripts otherwise showed similar expression patterns throughout the life-cycle (Fig. 6). Transcripts were found in all stages examined. The level of the SOD mRNAs increased slightly as the life-cycle progressed through the late larval stages to mature adulthood.

Protection trial

Lambs vaccinated with recombinant *H. contortus* SODs had modestly but significantly lower (17% reduction; $P < 0{\cdot}05$, analysis of variance) final worm burdens compared to the control group (Table 1). The faecal egg output from lambs immunized with the recombinant SODs tended to be higher than the controls throughout the experiment and this effect was significant ($P < 0{\cdot}05$) at days 26 and 28 post-infection (Fig. 7).

DISCUSSION

This study has shown that a gastrointestinal nematode, *H. contortus*, in common with other helminth parasites, expresses two distinct Cu/Zn SODs, namely a cytosolic and an extracellular form, the latter form of which may be excreted/secreted. RT–PCR analyses indicated that mRNA transcript abundance for both SODs increased as the life-cycle progressed and that SODc was approximately 6 times more abundant than SODe in all life-cycle stages. As far as the authors are aware, SOD has never been evaluated as a potential protective antigen against helminth infection although its potential in this area has been projected on several occasions.

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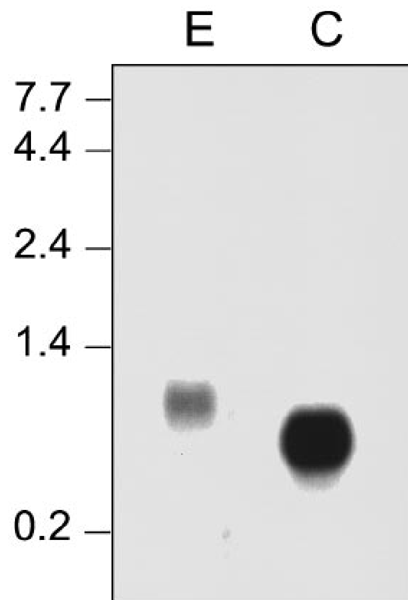

Fig. 5. Northern blot analysis of equal amounts of mRNA from 11-day-old adult parasites hybridized with SODc (C) and SODe (E) specific probes. The sizes of the RNA molecular weight markers are shown in kilobases.

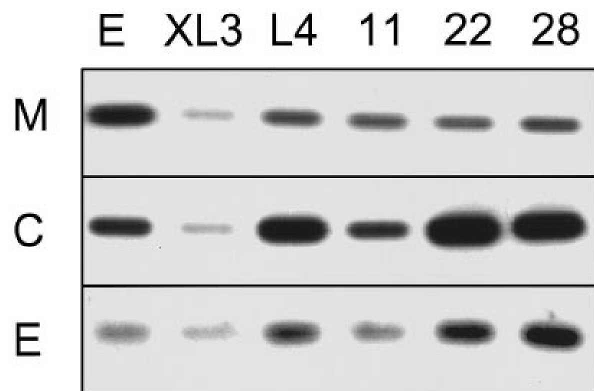

Fig. 6. Temporal expression of SODe and SODc *Haemonchus contortus*, determined using semi-quantitative RT–PCR. The figure shows an example of the autoradiographic data obtained for 1 reaction for each gene. Exposure time for the SODe blot (E) was 5 times that of the SODc (C) and myosin (M) blots. Stages examined were eggs (E), exsheathed L3 (XL3), 4th larval stage (L4), immature adult (11) and mature adult (22 and 28) where the numbers refer to days post-infection.

Here, vaccination of lambs with a combined recombinant SODc/SODe protein preparation resulted in a small, but significant, reduction in worm burdens 35 days after a single challenge infection with *Haemonchus*.

Getzoff *et al*. (1989) compared the primary sequences of 16 Cu/Zn SODs and identified 23 invariant residues required for enzyme structure and function. The predicted *H. contortus* Cu/Zn SOD sequences differed from these at 1 amino acid. In *H. contortus* SODc, leucine-107 is replaced with isoleucine, a functionally equivalent residue. *H. contortus* SODc and SODe showed 48% amino acid identity over their core sequences, an unusually low level of identity compared to that described for the cytoplasmic and extracellular SODs from the filarial nematodes *Brugia malayi* (88%; Tang *et al*. 1994) and *Onchocerca volvulus* (71%; Henkle *et al*. 1991), but similar to the level of identity found between the human cytoplasmic and extracellular SODs (38%; Tang *et al*. 1994).

Table 1. Worm burdens in the protection trial

(The 17% reduction in final worm burden in lambs vaccinated with recombinant SODc and SODe compared with the unvaccinated controls was significant ($P < 0{\cdot}05$, 2-sample Student's *t*-test).)

Group	Final worm burden	Percentage males	Mean daily faecal egg output (epg)
Controls	3655	51	4492
Vaccinates	3040	52	6008

Phylogenetic analysis confirmed the evolutionary proximity of the *H. contortus* SODs with their *C. elegans* counterparts and distinct from the distinct grouping of filarial SODs. The divergence of the filarial extracellular SODs from their cytosolic counterparts coincided with the divergence of the filarial cytosolic SODs from *C. elegans* cytosolic SOD (James, 1994).

Variant SODe sequences isolated may be ascribed to a multi-gene family, or may reflect the presence of different SODe alleles within the parasite population. The divergence of SODe sequences may be driven by external factors such as elements of the host immune response, and may indicate that SODe plays an important role in *H. contortus* survival in the host. In common with other nematodes (Tang *et al*. 1992) the *H. contortus* extracellular SOD lacked a positively charged C-terminal extension which mediates binding to proteoglycans on cell surfaces in the mammalian extracellular enzyme. Signal peptide cleavage at either cystine-24 or alanine-33 is only predicted and could be confirmed by N-terminal sequence analysis of the mature SODe. If the latter was purified from ES material, this analysis would either confirm that SODe is specifically released from the parasite *in vitro* or that ES SOD activity results from non-specific leakage of SOD from the parasite during culture.

The polyclonal antisera raised to recombinant SODc and SODe were unable to clearly differentiate the 2 SOD gene products in parasite extracts. However, only anti-HCSODe clearly recognized components in ES material, possibly indicating that the SODe gene product is the predominant SOD in ES and is in some way antigenically distinct. This

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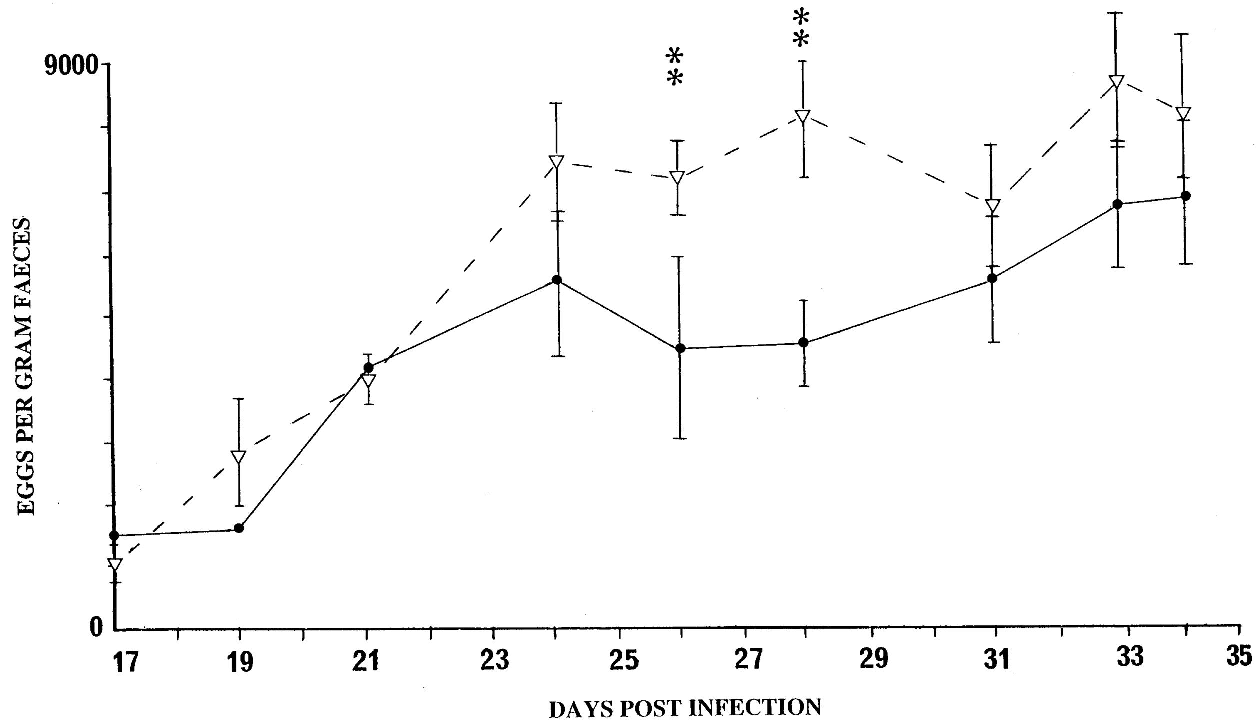

Fig. 7. The faecal egg output from lambs vaccinated with recombinant SODs (▽---▽) and unvaccinated controls (●—●).

interpretation would support the view that SODe is secreted from the parasite but would require confirmation by further analysis of ES SOD, N-terminal sequence analyses being particularly pertinent. The specific 16·8 kDa band detected in ES is smaller than the product size predicted after signal peptide cleavage although the discrepancy ($\sim$ 1 kDa) most likely reflects limitations in the precision of molecular weight determinations in gels using broad molecular weight range markers for comparison. The single reacting band in S1 extracts is consistent in size with the expressed recombinant protein indicating that this could be a non-glycosylated version of SODc. The cytosolic SOD encoding cDNA from *Brugia malayi* also predicted a single site for glycosylation and deglycosylation experiments showed that this was not occupied (Tang *et al.* 1994). The zone of reactivity at 19·8 kDa may represent glycoforms of SODc or SODe while the sizes of the ES products suggest that ES SOD is monomeric. The zone of reactivity evident between 32 and 37 kDa in S3 with both antisera may represent a dimeric form of the enzyme with the specific function of protecting membranes from oxidative free-radicals.

SOD was distributed in *H. contortus* in a similar manner to that reported for *Dirofilaria immitis* (Callahan *et al.* 1993) except that specific, though intermittent, staining was evident in the uterine tract. The latter may indicate that SOD is important at a particular stage of egg production. In *H. contortus* the predominant staining of the muscle cells of the body walls may support the role of SOD in protection of the lateral nerve tissue (Callahan *et al.* 1993); alternatively the high level of SOD in this region may simply reflect a high level of oxidative metabolism in these cells associated with contraction of the body wall and locomotion. *H. contortus* SOD was detected in the pharynx, possibly indicating secretion to the exterior, although consistent staining of the secretory glands was not observed. However, detection of SOD in *H. contortus* ES by Western blotting here and enzyme activity determinations (D. P. Knox, unpublished observations) is indicative of external release.

If SOD has an important role to play in parasite survival then it may have potential as a protective immunogen for vaccine production. Here, vaccination of lambs with recombinant versions of *Haemonchus* SODs prior to homologous challenge reduced final worm burdens but increased egg output when vaccinates and controls were compared. It is unclear how the reduction in final worm burdens was brought about; antibody-mediated inhibition of protective parasite SODs in vaccinates may have resulted in increased free-radical-mediated damage of parasites. The increase in egg output in vaccinated lambs is paradoxical, perhaps explicable by parasites favouring egg production in the short term at the cost of longevity. The sex ratio of the surviving worms recovered from individual lambs did not indicate that vaccination was more adverse to the female worms although estimations of worm biomass may alter this view.

This is the first report of the evaluation of SODs as potential protective antigens against any helminth

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infection, and the results indicate that these antigens merit further investigation. The recombinant SODe used here was not enzymatically active and antibodies raised may not inhibit native enzyme function, a property which may be essential to achieve maximum effect given the protective roles proposed for extracellular SODs in parasitic helminths. Also, the predominantly systemic antibody response stimulated here may be less effective than a regime which stimulates local immune responses in the host gastric mucosa. The model infection regime used, although quite standard, is atypical given that *H. contortus* infections are usually acquired by the regular ingestion of contaminated pasture over a period of time, a situation better reflected experimentally by trickle challenge regimes.

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