Characterization and Molecular Cloning of a Cu/Zn Superoxide Dismutase from the Human Parasite *Onchocerca volvulus*

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Evidence suggests that the helminth antioxidant enzyme superoxide dismutase (SOD) may play a role in parasite's defense against the cellular immune mechanisms of the host. In order to investigate this for the human parasite *Onchocerca volvulus*, the enzyme activity was characterized, the release of SOD by the parasite was examined, and a complete cDNA encoding the *O. volvulus* SOD was identified. The SOD activity in adult *O. volvulus* was found to be 8.1 ± 4.2 U/mg of protein. A Cu/Zn-containing enzyme was demonstrated by its sensitivity towards cyanide, azide, and hydrogen peroxide. Isoelectric focusing, combined with an enzyme activity assay, revealed two activities at pI 6.8 and 7.6, with both activities inhibited by KCN. Adult parasites, maintained in vitro, released SOD into the culture medium, which was detected by enzyme activity. In parallel, lactate production was measured to ensure the viability of the parasite. Oligonucleotides (based upon conserved sequences in the SOD genes of other organisms) and the polymerase chain reaction were used to identify a portion of the SOD gene from *O. volvulus* genomic DNA. A cDNA library was constructed in lambda unizapII and screened with the genomic polymerase chain reaction fragment. A complete cDNA encoding the Cu/Zn SOD was identified, and its nucleotide sequence was determined. Southern blot hybridization experiments indicated that the Cu/Zn SOD is encoded by a single-copy gene with at least one intron.

Onchocerca volvulus is a pathogenic human parasite which, like many other filarial parasites, lives close to host effector cells and is not damaged by the host. An understanding of the mechanisms which permit these parasites to evade the effects of the host immune response is crucial. It has been suggested that one aspect of parasite defense may be the production of enzymes which are capable of responding to and inactivating host cytotoxic molecules (7). Among the candidates for this role is the antioxidant enzyme superoxide dismutase (SOD). The normal function of SOD is to protect cells from oxidant-mediated damage due to the O_2^- radical, which is a by-product of a variety of metabolic reactions. Activated phagocytes including eosinophils and neutrophils use, at least in part, free-radical-mediated mechanisms generating O_2^- , H_2O_2 , and $\cdot OH$ for antiparasitic (20–22) and antimicrobial (16, 35) activity. The antiinflammatory properties of SOD and its ability to inhibit these killing mechanisms have been demonstrated previously (19, 24, 27). Therefore, it has been speculated that in filarial parasites, SOD may protect the parasite from the host response and ameliorate tissue inflammation in addition to its normal cellular function.

Interestingly, current evidence suggests that SOD in parasites such as *Trichinella spiralis* (25), *Taenia taeniaeformis* (17), and *Schistosoma mansoni* (30) is secreted or in some way released into culture fluid in vitro and possibly into the parasitic environment in vivo. In addition, the nucleotide sequence of a *S. mansoni* SOD cDNA revealed that the encoded protein contained a predicted hydrophobic signal sequence (30). To assess the situation for the human parasite *O. volvulus* and to help establish the precise role of SOD in the protection of filarial parasites, we have characterized SOD enzyme activity from *O. volvulus*, maintained live worms in vitro to examine SOD release into culture medium,

and cloned a partial genomic DNA fragment and a complete cDNA fragment encoding a SOD. The cloned cDNA has allowed us to examine the predicted amino acid sequence of the *O. volvulus* SOD for the presence of a signal peptide and to examine the *O. volvulus* genome for SOD gene organization and gene copy number.

(Part of this research was conducted by Eva Liebau in partial fulfillment of the requirements for a Ph.D. from University of Hamburg, Hamburg, Germany).

MATERIALS AND METHODS

Parasites. Adult *O. volvulus* worms were isolated and prepared as previously described (29, 34). Briefly, nodules containing the parasite were isolated from Liberian patients and shipped on ice to the laboratory within 24 h. Worms were isolated from the nodules by microdissection and were then incubated in RPMI 1640 with 0.5% collagenase (*Clostridium histolyticum*) to eliminate host tissue. The collagenase digestion was performed in 3 to 10 ml of the RPMI 1640-collagenase solution per nodule, in a 34°C shaking incubator for up to 24 h, changing the solution every 8 to 10 h. Finally, the worms were examined microscopically for purity from host material.

In vitro maintenance of *O. volvulus*. The worms could survive in RPMI 1640 under a gas phase of 5% O_2 , 5% CO_2 , and 90% N_2 for up to 4 weeks. Each female worm was maintained in 2 ml of medium, which was changed daily. Production and release of lactate, the main product of glucose metabolism in *O. volvulus* (36), was determined daily as a parameter of viability. Proteins secreted into the spent medium were harvested for 5 days, while the lactate production assay was continued for more than 2 weeks. The spent medium was concentrated by ultrafiltration (Immersible CX-10; Millipore) and used in SOD activity assays.

Preparation of parasite extract and SOD enzyme assay. Adult female *O. volvulus* were homogenized by hand in 3

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volumes of 50 mM Tris-HCl (pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at $100,000 \times g$ for 60 min, and the supernatants were used for the enzymatic assays. The activity of SOD in the parasite extract or the spent medium was measured using the ferricytochrome *c* reduction assay by the method of Flohé and Ötting (10), using human erythrocyte SOD as the standard. One unit of SOD activity is defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50% under the conditions specified. To characterize the type of SOD enzyme, the assay was performed in the presence of 1 mM KCN or 5 mM sodium azide or inhibition by H_2O_2 was demonstrated by the method of Asada et al. (2), as previously reported (1).

Protein determination. Protein content was determined as described previously by Bradford (6) with bovine serum albumin as the standard.

Isoelectric focusing. Isoelectric focusing was performed on a polyacrylamide gel with a pH range of 4.0 to 6.5 according to the instructions supplied by the manufacturer (Pharmacia). To determine the pH gradient profile, a microelectrode and a pI calibration kit were used. The gels were stained for SOD activity by incubation in Nitro Blue Tetrazolium as described by Beauchamp and Fridovich (4).

Chemicals. SOD from human erythrocytes and cytochrome *c* type III were purchased from Sigma Chemie, Deisenhofen, Germany. Ampholine PAG plates and a pI calibration kit were obtained from Pharmacia, Freiburg, Germany. Nitro Blue Tetrazolium and phenylmethylsulfonyl fluoride were from Serva Feinbiochemica, Heidelberg, Germany. RPMI 1640 was from Gibco, Karlsruhe, Germany. Collagenase was from Boehringer, Mannheim, Germany. All other chemicals were of reagent grade and obtained from Merck, Darmstadt, Germany.

Preparation of RNA and DNA. Adult *O. volvulus* worms were homogenized in guanidinium thiocyanate and layered on a CsCl step gradient, and RNA and DNA were prepared as previously described (8). Poly(A)⁺ RNA was isolated by oligo(dT) chromatography (28). The RNA quality was examined by in vitro translation in a rabbit reticulocyte lysate (Boehringer) with [³⁵S]methionine (23) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

PCR. Using the primers which were constructed based upon conserved sequences in the SOD cDNAs of other organisms and total genomic DNA from *O. volvulus*, the SOD genomic fragment was amplified by 25 cycles of polymerase chain reaction (PCR) at 95°C for 2 min, 42°C for 2 min, and 72°C for 2 min (26). The amplified products were analyzed on agarose gels, purified, subcloned into pBlue-scribe, and sequenced in the same manner as the cDNAs. Reagents and Amplitaq were purchased from Perkin-Elmer Cetus, Überlingen, Germany.

cDNA library construction. cDNAs were synthesized from 5 µg of poly(A)⁺ RNA, initiating with an oligo(dT) primer containing an *Xho*I restriction enzyme recognition site (12), using reagents and enzymes purchased from Stratagene. The cDNAs were treated to produce blunt ends, and *Eco*RI adapters were added. The cDNAs were digested with *Xho*I and then separated from the *Xho*I linker-primer by Sepharose CL-4B chromatography. These cDNAs were ligated to *Eco*RI-*Xho*I-cleaved lambda unizapII, and the reaction products were packaged into bacteriophage, using packaging extracts from Stratagene. The unamplified library contained 2.7×10^6 recombinant phage, with only 1% of the PFU lacking an *O. volvulus* cDNA insert.

Identification of the cDNA. The isolated SOD genomic

TABLE 1. Sensitivity of the SOD from *O. volvulus* and human erythrocytes to various inhibitors^a

Inhibitor (concn)	Inhibition rate (%)	
	<i>O. volvulus</i>	Human erythrocyte ^b
KCN (1 mM)	65	100
NaN ₃ (5 mM)	36	30
H ₂ O ₂ (1 mM)	42	35

^a Standard enzyme activity assays were performed in the presence and absence of the inhibitor. For H_2O_2 , the enzyme preparations were preincubated for 60 min at 25°C.

^b Data taken from the work of Arias and Walter (1).

DNA PCR fragment was labeled with [³²P]dATP by the random primer method (9) and used as a hybridization probe. The cDNA library was screened by a standard plaque hybridization assay (5). The SOD cDNA initially identified was later used as a hybridization probe in a second round of screening to isolate larger cDNAs. Eight *O. volvulus* SOD cDNAs were identified and sequenced. One of these cDNAs contained the complete coding region and 5' and 3' nontranslated regions.

DNA sequencing. The nucleotide sequences of the cDNAs were determined by performing the Sanger dideoxy-chain termination reactions on double-stranded DNA (28), using [³⁵S]dATP and Sequenase (United States Biochemical Corporation, Cleveland, Ohio). In addition to obtaining the cDNA sequences using the standard forward and reverse oligonucleotides, the complete cDNA was cleaved at an internal *Eco*RI site and recloned into pBluescribe, and the sequences of both strands were determined.

Genomic Southern blot analysis. *O. volvulus* and human genomic DNAs (10 µg per lane) were digested with various restriction enzymes (Boehringer), electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose paper (31). The cloned cDNAs used as hybridization probes were labeled by the random primer method (9). The filters were hybridized at 65°C for 16 h, washed, and autoradiographed using conditions previously described (14).

Nucleotide sequence accession number. *O. volvulus* Cu/Zn SOD mRNA has been assigned EMBL accession no. X57105.

RESULTS

Characterization of the *O. volvulus* SOD activity. The specific activity of SOD in total worm extract was determined and was found to be 8.1 ± 4.2 U/mg of protein ($n = 4$). This SOD was classified as a Cu/Zn-containing enzyme on the basis of its sensitivity toward cyanide, azide, and hydrogen peroxide. Inhibition rates and inhibitor concentrations are shown for the enzyme from *O. volvulus* and compared with values determined for the Cu/Zn-containing SOD from human erythrocytes (18) (Table 1). Isoelectric focusing, combined with an enzyme activity assay performed by staining the gel with Nitro Blue Tetrazolium, revealed two activities at pI 6.8 and 7.6 (Fig. 1A). Both of these activities decreased in the presence of 0.5 mM KCN (Fig. 1B), and 2 mM KCN abolished the activity completely.

In vitro maintenance of adult worms to assay SOD release into culture medium. When live female *O. volvulus* worms were maintained in culture medium, SOD activity could be detected in the medium. It was determined that in the spent medium, there was 0.72 ± 0.28 U per worm per day. When this activity is recalculated on the basis of the total amount

FIG. 1. Identification of Cu/Zn SOD activity in the *O. volvulus* extract and in the spent medium. The activity is characterized in the presence (B) and absence (A) of the Cu/Zn SOD inhibitor, KCN, at a concentration which will only partially inhibit enzyme activity. The electrophoresis and staining procedures are described in Materials and Methods.

of released proteins, rather than the total amount released per individual worm per day, there were 13.3 ± 6.9 U of activity per mg of protein ($n = 4$). During maintenance, the quantity of *O. volvulus* proteins released into the culture medium, including SOD, declined rapidly. In a representative experiment, the amount of SOD released into the medium decreased from 0.58 to 0.15 to 0.09 U per worm over 3 days. To demonstrate that the release of SOD does not derive from leakage of damaged worms, the viability and survival of the worms were examined by determining lactate production over an extended period. Experiments were conducted on individual worms to avoid having a mixed population of living and dead parasites. Lactate production and release declined after 3 to 5 days but subsequently continued at this reduced rate for an additional 3 weeks.

The SOD activity found in the spent medium was characterized and was also found to contain activities with pIs at 6.8 and 7.6 (Fig. 1) which demonstrated the inhibition characteristics of a Cu/Zn-containing enzyme. In the presence of 0.5 mM cyanide, the SOD activity observed by staining the gel decreased (Fig. 1B) relative to that of the untreated enzyme (Fig. 1A), while higher concentrations of cyanide completely abolished enzyme activity and resulted in no observable bands in this gel activity assay (data not shown).

Identification of the genomic SOD PCR fragment. A comparison of the amino acid sequences of known SODs from various species (3, 30) revealed four regions that are strongly conserved among species. These amino acid stretches, G P H F N P, V G D L G N, D D L G R, and G P R L A C, were used in designing oligonucleotides which would be likely to bind to the *O. volvulus* SOD gene. When an amino acid was not absolutely conserved or when various codons had been used to encode the same amino acid, preference was given to the *S. mansoni* nucleotide sequence. Various oligonucleotide pairs were used for the PCR, since the SOD gene structure in *O. volvulus* was not known and an intron could be present between the oligonucleotide binding sites. The PCR SOD genomic fragment that was identified contained a 263-bp intron, which is shown in Fig. 2. Flanking the intron were the consensus splice signals (5'-GT...AG-3') (28). The nucleotide sequence and the deduced amino acid se-

quence of the PCR fragment, excluding the intron, resembled an SOD, although this partial sequence was insufficient to make a firm conclusion. However, this fragment was used to screen the lambda unizapII cDNA library.

Identification of the *O. volvulus* SOD cDNAs. The initial *O. volvulus* SOD cDNA (A3A cDNA) was identified in the lambda unizapII cDNA library with the labeled SOD genomic PCR fragment using low-stringency hybridization conditions. The phagemid containing the SOD cDNA (pA3A) was recovered from lambda unizapII by the automatic excision process as described by the manufacturer (Stratagene), and the nucleotide sequence of the pA3A insert was determined. The insert was 590 bp and showed significant nucleotide and deduced amino acid sequence homologies to the Cu/Zn SODs of other organisms. The pA3A cDNA insert was then used as a probe to search for a complete cDNA containing the entire coding region. Seven additional *O. volvulus* SOD cDNAs were identified, and their nucleotide sequences were determined. One of these, pIEL-12, was 643 bp and contained the complete coding region and both 5' and 3' nontranslated regions. This sequence is shown in Fig. 2. There were four positions within the nucleotide sequences of these eight cDNAs which varied, only one of which resulted in an amino acid change. At position 128, a G in the nucleotide sequence of seven of the cDNAs was found to be an A in one cDNA, resulting in an isoleucine at this position rather than a valine. This conservative amino acid substitution is consistent with an SOD sequence because the human sequence contains an isoleucine at this position, while the schistosome sequence contains a valine. Two other nucleotide differences were observed within the coding region, an A rather than a G at position 139 and a T rather than a C at position 235. Both of these are changes in the third nucleotide position within a codon and do not result in an amino acid difference. In addition, the length of the 3' nontranslated region differed between the clones. While five cDNAs contained the sequence shown in Fig. 2, three ended at the stretch of A's at position 540. One of the cDNAs with the longer 3' nontranslated region contained an additional G at position 631 within the final run of A's. This 3' nontranslated region does not contain a consensus polyadenylation signal sequence.

Analysis of the SOD deduced amino acid sequence. Identification of the *O. volvulus* SOD cDNA allowed the analysis of the deduced amino acid sequence. The predicted coding region contains 158 amino acids, and the calculated molecular mass is 16,339 Da. When this sequence is compared with the amino acid sequences of the SODs from other organisms (Fig. 3), a significant amount of sequence identity is found. When gaps were used in all of the sequences to improve the alignment, the homologies are 56% for the *O. volvulus* and human SOD and 38% for the *O. volvulus* and *S. mansoni* SOD. Most importantly, the amino acids that are known to be required for enzyme activity and those which are involved in metal binding are conserved (3). The histidine and aspartate residues (His-46, His-48, His-63, His-71, His-80, His-120, and Asp-83) involved in copper and zinc binding (3, 11, 33) are boxed in Fig. 3. The two cysteines (Cys-57 and Cys-149) which form an intrasubunit disulfide bridge in the Cu/Zn SODs are also conserved and are boxed. The arginine (Arg-146) which is normally at the entrance of the active site and which directs the superoxide anion to the copper atom (3, 11) is also present. Beyond position 16 in the *O. volvulus* and human sequences and position 47 in the *S. mansoni* sequence, the proteins are similar in all three of these species; the N-terminal portions, however, are not

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                M S T N A I A V L R G      11
TGAGCTTGAAACCGGTTGTATTATCATGAGTACAAACGGATAGCAGTATTGCGTGCG      58

D T V S G I I R F K Q D K E G L P T T V      31
GATACTGTTAGTGGAATTATTCGATTTAAACAGGACAAGGAAGGCTTACCAACAACCGTT      118

T G E V K G L T P G L H G F H I H Q Y G      51
ACTGGTGAAGTCAAAGGTTTGACTCCTGGTTTACATGGTTTTTCATATTCATCAGTATGGT      178

D T T N G C I S A ↓ G P H F N P Y N K T H      71
GACACGACAAATGGGTGCATTTCTGCGGGTCCGCATTTCAATCCTTACAATAAAACCCAT      238

G D R T
GGCGATCGAACT gttagttttatctccagaggataagcgaaaacttgaaatc

tatgctagttttattattttaattgctgaaataatggctgtcttaaatgttta

taataggacgaactatgtaacataaattgaaaaattatttgaattctgataaa

ttatgacttatgttaaaaagtttcaactattttatgatagaatcaaataattct

aatgaaaattagtggttttttttattgtctttcattcgtaattcacaaactaat

                D E I R H V G D L G N ↓ I E A G A      91
tttttag GATGAAATAAGACATGTTGGTGATCTTGGAAATATTGAAGCTGGAGCC      298

D G T A H I S I S D Q H I Q L L G P N S      111
GATGGTACTGCTCACATTAGCATTTCCGATCAACATATACAGTTGCTGGGTCCGAATTCG      358

I I G R S I V V H A D Q D D L G K G V G      131
ATAATTGGCCGTTCAATTGTTGTACATGCTGATCAGGATGATCTCGGAAAAGGAGTCGGC      418

A K K D E S L K T G N A G A R V A C G I      151
CGGAAAAGGATGAAAGCCTGAAAACCTGGTAATGCTGGTGCCTGTTGCATGCGGTATT      478

V A I G A A S *      158
GTCGTATTGGTGCTGCTTCCTAAATCGATTTTCTTTTGTTCATGAATTTCTCTCTCTAA      538

AAAAGAAAATGTTTGTAGAATATAATGATTTGTTGAATGACATCCTTCATGCTCTTAGTG      598

TTCCTATCTATTCTGTGAAATTTCTTTGTAAAAA          643

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FIG. 2. Nucleotide sequence and predicted amino acid sequence of the complete *O. volvulus* Cu/Zn SOD cDNA and nucleotide sequence of a partial *O. volvulus* SOD genomic DNA PCR fragment. The sequence of the intron, located within the sequenced genomic DNA region flanked by the arrows, is in lowercase type. The consensus splice junction signals are in boldface type, and the two *EcoRI* sites are underlined.

similar. The N terminus of the *S. mansoni* sequence is quite distinctive and contains a predicted hydrophobic signal peptide. This is obviously lacking in the identified *O. volvulus* SOD and its N terminus resembles that of the human cytosolic SOD.

Genomic Southern blot analysis. Genomic Southern blot analysis was performed to demonstrate the origin of the cDNA and to partially map the *O. volvulus* SOD gene. Since the adult parasites are always obtained from human host tissue, it is possible that the parasite RNA preparation, which is used for cDNA library construction, is contaminated with human RNA. To exclude the possibility that the SOD cDNAs identified were derived from humans, the cDNA insert from pA3A was used to probe a genomic DNA Southern blot, containing both human and *O. volvulus* DNAs, at high stringency. As shown in Fig. 4, the pA3A cDNA insert hybridized specifically with the *O. volvulus* DNA and not to the human DNA. This substantiates the sequence comparison and confirms that the cDNAs reported here are derived from *O. volvulus*. The *O. volvulus* digests show a simple pattern of hybridization in which all of the bands can be explained if there is only a single-gene copy on the basis of the restriction map of the cDNA and the intron. The *EcoRI* digest is the most informative in defining a single gene, because one *EcoRI* site was found within the cDNA sequence and another was found within the sequenced intron. The exact distance between these mapped sites is 226 bp. This fragment is observed as a light band in the *O.*

volvulus *EcoRI* (lane E) digest. If there are additional introns within the gene, they seem not to contain *EcoRI* sites, since the remaining two bands in this lane can be explained as the 5' and 3' halves of the gene flanking the small *EcoRI* fragment. When this Southern filter was reprobbed with only the 3' half of the cDNA (data not shown), only the 1.2-kb and 226-bp fragments hybridized to the probe. Therefore, the 3.5-kb *EcoRI* fragment contains the 5' portion of the gene, the 226-bp fragment lies in the middle, and the 1.2-kb band contains the 3' end. In addition, when a partial genomic DNA digest was probed with the cDNA, there was no evidence for tandemly repeated genes. This fact and the absence of unexplained hybridizing fragments indicates the presence of only one gene encoding this Cu/Zn SOD within the *O. volvulus* genome. The digest with *HaeIII* supports this because one *HaeIII* site was found within the cDNA coding sequence, therefore predicting two bands. In the *HaeIII* digest (lane H), the 1.8-kb fragment contains the 3' end of the gene and the 6.0-kb fragment contains the 5' portion of the gene. The entire coding region within the pA3A cDNA insert appears to lie on a 4.0-kb *HindIII* (lane Hd) fragment.

DISCUSSION

The ability of adult helminth parasites to circumvent the immune responses of the host could be attributed to a number of possible mechanisms. Among the more com-

SM	M T V Y S Y L V I L F I L L D N Y C S A Y G Y G Y S Y Y H R	30
HU		
OV	M A T - K A V C V L K G D G P V Q G I I N F E Q - K	24
SM	M S T - N A I A V L R G D T - V S G I I R F K Q D K	24
	R H F D P A I A S F T - - - K - E - P Y I G A V W F T Q - H	54
HU	E S N G P V K V W G S I K G L T E G - L H G F H V H E F G D	53
OV	E - G L P T T V T G E V K G L T P G - L H G F H I H Q Y G D	52
SM	G - D Y - M Y V N G S V A G L P P G K L L G T H V H R Y G G	82
HU	N T A G C T S A G P H F N P L S R K H G G P K D E E - R H V	82
OV	T T M G C I S A G P H F N P Y N K T H G D R T D E I - R H V	81
SM	L G N M C L E A G P H F N P F N Q R H - G P R H G Y P R H A	111
HU	G D L G N V T A D K D G V A D V S I E D S V I S L S G D H C	112
OV	G D L G N I E A G A D G T A H I S I S D Q H I Q L L G P N S	111
SM	G D L G N I R V G R G V A K F D F Y V T - I K G L G P F D	140
HU	I - I G R T L V V H E K A D D L G K G - - - G N E E S T K -	137
OV	I - I G R S I V V H A D Q D D L G K G V G A K K D E S L K T	140
SM	G F I G R A L V I H A N R D D L G R N - - - R D E G S R T T	167
HU	G N A G S R L A C G - V - I G I - A Q *	153
OV	G N A G A R V A C G I V A I G - A A S *	158
SM	G N S G P R L A C A T - - I G F R A P *	184

FIG. 3. Comparison of the amino acid sequences of *O. volvulus*, human, and *S. mansoni* SODs. *O. volvulus* (OV), human cytosolic (HU) (data from Sherman et al., 1983), and *S. mansoni* (SM) (data from Simurda et al. [30]) sequences are shown. The conserved amino acids required for enzyme activity, copper and zinc binding, and disulfide bond formation are boxed and are discussed in Results. The dashes indicate spaces introduced for optimal alignment of the sequences.

monly suggested are the production of (i) proteinase inhibitors which block complement activation and neutrophil chemotaxis (17, 32), (ii) factors which inhibit mast cell degranulation (18), and (iii) scavengers of free radicals and oxidants, such as SOD, catalase, and glutathione peroxidase

FIG. 4. Southern blot analysis of *O. volvulus* and human genomic DNAs probed with the pA3A SOD cDNA. Human DNA (H) was digested with *EcoRI* (E). *O. volvulus* DNA was digested with *EcoRI* (E), *HindIII* (Hd), and *HaeIII* (H). The molecular size markers are *HindIII*-digested lambda DNA.

(7). The work presented here is an attempt to begin to assess the validity of the third suggestion. More specifically, it is the groundwork for the subsequent analysis of the role of SOD in the host-parasite relationship.

It is known that normal, pathogenic, and surgical processes can result in the production of toxic free radicals. For example, these radicals are produced in normally functioning cells by oxidative enzymes such as xanthine oxidase and aldehyde oxidase, in the joints of patients with osteoarthritis, and in newly transplanted organs following reperfusion. Investigations of the potential of Cu/Zn SOD for the therapeutic treatment of inflammation due to pathogenic processes and surgery are under way. SOD has already been used to treat osteoarthritis (3) and to decrease the damage that occurs during tissue reperfusion following organ transplant (19, 24). These toxic radicals are also produced during the inflammatory response of phagocytes against bacteria and parasites. In the same way that SOD is used clinically as an antiinflammatory agent by administering purified or recombinant enzyme at the required site to avoid tissue damage, perhaps the parasites use SOD not only in its normal function but also as a way of protecting themselves against damage from the superoxide radical-producing phagocytes.

The localization of the SOD in these parasites then becomes a crucial issue if we speculate that it is being used as a scavenger of radicals generated by attacking phagocytes. The in vitro release of SOD by *O. volvulus* (presented here) and by *T. spiralis* (25) and *T. taeniaeformis* (17) is intriguing because it suggests but does not prove that SOD in these

parasites may be located at the surface or even actively secreted from the parasite. The facts that the protein encoded by the *S. mansoni* SOD cDNA contains a potential signal sequence (30) and that humans and animals infected with these parasites have fairly high antibody titers against the parasite SOD (17, 25, 30) support this idea. However, the *O. volvulus* SOD cDNA sequence was found not to encode a protein with a signal sequence. At this point, it is possible to speculate that there are two forms of the Cu/Zn SOD in these helminth parasites which are analogous to the human cytosolic (13) and extracellular (3, 15) SODs. The SOD reported from *S. mansoni* possesses a signal sequence like the human extracellular SOD, while the N terminus of the *O. volvulus* SOD described here resembles that of the cytoplasmic SOD. The functional roles of the human and the parasite cytosolic SODs would be similar, and both lack signal peptides. The human extracellular SOD and the signal peptide-containing enzyme from the parasites may have different functions. In humans, the extracellular SOD is found mainly in extracellular fluids such as plasma, lymph, and synovial fluid. This form of the parasite enzyme may function in parasite defense. The existence of two such forms of Cu/Zn SOD in any of these parasites has not yet been demonstrated. The possibility that the two proteins observed in the isoelectric focusing experiment (shown in Fig. 1) are these two forms is tantalizing, although the presence of both bands in both the parasitic extract and the culture medium argues against this. The hypothesis that another form of the Cu/Zn SOD exists in *O. volvulus*, which has a signal peptide and is similar to the schistosome enzyme, is being examined.

The enzymatic and sequence analysis of the *O. volvulus* SOD presented here revealed characteristics and a structure similar to that of cytosolic Cu/Zn SODs from other species. The activity, inhibition characteristics, and pI values are typical (3, 17). The difference between the two molecular forms, which has also been observed for other organisms (25), cannot be explained at this time. The calculated molecular mass of 16,339 Da is consistent with the monomer molecular mass. It is likely that the *O. volvulus* protein, like other Cu/Zn SODs, is active as a dimer with a molecular mass of approximately 32,000 Da (3). The molecular mass of the reported schistosome SOD, 20,300 Da, was slightly larger (30). The deduced primary structure of the *O. volvulus* protein is also consistent with the features of a protein with SOD activity expected on the basis of homology with other SODs and the absolute conservation of residues required for activity or secondary structure formation (3). The differences observed between the nucleotide sequences of the cDNAs, which do not alter conclusions about the deduced protein sequence, may be due to population variation (the library was constructed from RNA from 30 adult worms) due to the normal low-level frequency of point mutations or mistakes by the reverse transcriptase during cDNA synthesis. The latter idea seems unlikely because the changes either result in no amino acid change or a conservative one. There is also no evidence for multiple genes within the *O. volvulus* genome; therefore, the differences are probably best explained by variation within the adult worm population. The differences in the length of the 3' nontranslated regions could be due to genuine differences in the lengths of the mRNAs or the hybridization of oligo(dT) during first-strand cDNA synthesis to A-rich regions other than the poly(A) tail.

On the basis of these results, we are now examining the possibility of a second signal-peptide-containing Cu/Zn SOD

in *O. volvulus*. In addition, recombinant *O. volvulus* SOD will be prepared so that antiserum can be raised and then used to more precisely define the localization of this enzyme within the worm. Finally, it is possible that a comparison of the inhibition characteristics of the recombinant *O. volvulus* and recombinant human SODs may reveal a compound which can specifically inhibit the parasite enzyme or that non-cross-reactive antibodies may be useful in tackling the parasite's defense system.

ACKNOWLEDGMENTS

We thank Anke Lüchow for excellent technical assistance; Rolf Horstmann, Egbert Tannich, and Iris Bruchhaus for helpful discussions; and Peter Zipfel, Ulrich Dührsen, and Hans Müller-Eberhard for suggestions and review of the manuscript.

This work was supported, in part, by the Alexander von Humboldt Foundation and Deutsche Forschungsgemeinschaft WA395/5-1.

REFERENCES

1. Arias, A. E., and R. D. Walter. 1988. Plasmodium falciparum: association with erythrocytic superoxide dismutase. *J. Protozool.* 35:348-351.
2. Asada, K., K. Yoshikawa, M. Takahashi, Y. Marda, and K. Enmanji. 1975. Superoxide dismutase from blue-green alga *Plectonema boryanum*. *J. Biol. Chem.* 250:2801-2807.
3. Bannister, J. V., W. H. Bannister, and G. Rotilio. 1987. Aspects of the structure, function and applications of superoxide dismutase. *Crit. Rev. Biochem.* 22:111-180.
4. Beauchamp, C. O., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44:276-287.
5. Benton, D. W., and R. W. Davis. 1975. Screening lambda gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180-182.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
7. Callahan, H. L., R. K. Crouch, and E. R. James. 1988. Helminth anti-oxidant enzymes: a protective mechanism against host oxidants? *Parasitol. Today* 4:218-225.
8. Chirgwin, J. M., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
9. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
10. Flohé, L., and F. Ötting. 1984. Superoxide dismutase assays. *Methods Enzymol.* 105:93-104.
11. Getzoff, E. D., J. A. Tainer, P. K. Weiner, P. A. Kollman, J. S. Richardson, and D. C. Richardson. 1983. Electrostatic recognition between superoxide and copper, zinc superoxide dismutase. *Nature (London)* 306:287-290.
12. Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.
13. Halliwell, R. A., F. R. Masiarz, R. C. Najarian, J. P. Puma, M. R. Quiroga, A. Randolph, R. Sanchez-Pescador, C. J. Scandella, B. Smith, K. S. Steimer, and G. T. Mullenbach. 1985. Human Cu/Zn superoxide dismutase cDNA: isolation of clones synthesizing high levels of active or inactive enzyme from an expression library. *Nucleic Acids Res.* 13:2017-2034.
14. Henkle, K. J., G. A. Cook, L. A. Foster, D. M. Engman, L. A. Bobek, G. D. Cain, and J. E. Donelson. 1990. The gene family encoding eggshell proteins of *Schistosoma japonicum*. *Mol. Biochem. Parasitol.* 42:69-82.
15. Hjalmarsson, K., S. L. Marklund, A. Engstrom, and T. Edlund. 1987. Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 84:6340-6344.
16. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic

- polymorphonuclear leukocytes. *Semin. Hematol.* **12**:117-142.
17. **Leid, R. W., and C. M. Suquet.** 1986. A superoxide dismutase of metacestodes of *Taenia taeniaeformis*. *Mol. Biochem. Parasitol.* **18**:301-311.
 18. **Mazingue, C., D. Camus, J.-P. Dessaint, M. Capron, and A. Capron.** 1980. In vitro and in vivo inhibition of mast cell degranulation by a factor from *Schistosoma mansoni*. *Int. Arch. Allergy Appl. Immunol.* **63**:178-189.
 19. **McCord, J. M.** 1985. Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* **312**:159-163.
 20. **Murray, H. W.** 1981. Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* **153**:1302-1315.
 21. **Murray, H. W., C. W. Juangbhanich, C. F. Nathan, and Z. A. Cohn.** 1979. Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. *J. Exp. Med.* **150**:950-964.
 22. **Nathan, C., N. Nogueira, C. Juangbhanich, J. Ellis, and Z. Cohn.** 1979. Activation of macrophages in vivo and in vitro. Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. *J. Exp. Med.* **149**:1056-1068.
 23. **Pelham, H. R. B., and R. S. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* **67**:247-256.
 24. **Petrone, W. F., D. K. English, L. Wong, and J. M. McCord.** 1980. Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma. *Proc. Natl. Acad. Sci. USA* **77**:1159-1163.
 25. **Rhoads, M. L.** 1983. *Trichinella spiralis*: identification and purification of superoxide dismutase. *Exp. Parasitol.* **56**:41-54.
 26. **Saiki, R., S. Scharf, F. Faloona, K. Mullis, G. Horn, H. Erlich and N. Arnheim.** 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354.
 27. **Salin, M. L., and J. M. McCord.** 1975. Free radicals and inflammation. Protection of phagocytosing leukocytes by superoxide dismutase. *J. Clin. Invest.* **56**:1319-1323.
 28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 29. **Schulz-Key, H., E. J. Albiez, and D. W. Büttner.** 1977. Isolation of living adult *Onchocerca volvulus* from nodules. *Trop. Med. Parasitol.* **28**:428-430.
 30. **Simurda, M. C., H. van Keulen, D. M. Rekosh, and P. T. LoVerde.** 1988. *Schistosoma mansoni*: identification and analysis of an mRNA and a gene encoding superoxide dismutase (Cu/Zn). *Exp. Parasitol.* **67**:73-84.
 31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
 32. **Suquet, C. M., C. Green-Edwards, and R. W. Leid.** 1984. Isolation and partial characterisation of a *Taenia taeniaeformis* metacestode proteinase inhibitor. *Int. J. Parasitol.* **14**:165-172.
 33. **Tainer, J. A., E. D. Getzoff, J. S. Richardson, and D. C. Richardson.** 1983. Structure and mechanism of copper, zinc superoxide dismutase. *Nature (London)* **306**:284-287.
 34. **Walter, R. D.** 1988. Preparation and shipping of *Onchocerca volvulus* material. *Trop. Med. Parasitol.* **39**:447.
 35. **Weiss, S. J., M. B. Lampert, and S. T. Test.** 1985. Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science* **222**:625-628.
 36. **Wittich, R. M., and R. D. Walter.** 1987. *Onchocerca volvulus*: partial glucose catabolism via fumarate and succinate. *Exp. Parasitol.* **64**:517-518.