

Angiogenic activity of *Onchocerca volvulus* recombinant proteins similar to vespid venom antigen 5

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Abstract

Although the mechanisms underlying the host inflammatory response in ocular onchocerciasis have been examined, the role of particular parasite proteins in this process remains largely unexplored. Recently, it was found that one of the most abundant expressed sequence tags in *Onchocerca volvulus* infective larvae encoded a protein with similarities to a component of vespid venom. This clone was designated *O. volvulus* Activation associated Secreted Protein -1 (*Ov-asp*-1). We report the characterization of three members of a family of proteins, designated the *Ov*-ASP family, of which *Ov*-ASP-1 is a member. Sequence based and phylogenetic analyses suggest that these proteins form a filarial specific protein family related to both the vespid venom antigen 5 and the vertebrate CRISP/Tpx family of proteins. The three members of the *Ov*-ASP family exhibit distinct patterns of expression in the life cycle of *O. volvulus*. Genomic Southern blot analyses indicate that several genes encoding sequences related to the *Ov-asp* family are present in the genome of *O. volvulus*. Recombinant proteins expressed from full length cDNAs encoding two members of the *Ov-asp* family were found to induce an angiogenic response after injection into corneas of naive mice, and vessel formation was associated with only minor inflammatory cell infiltration. These data suggest that *Ov*-ASP proteins may directly induce an angiogenic response and may therefore contribute to corneal neovascularization in onchocercal keratitis. © 2000 Elsevier Science B.V. All rights reserved.

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Abbreviations: Ag5, vespid antigen 5; CRISP, cysteine-rich secreted protein; MBP, Maltose binding protein; *Ov*-ASP, *Onchocerca volvulus* activation associated secreted protein; *Ov*-PDI, *Onchocerca volvulus* protein disulfide isomerase; Tpx, testis-specific protein.

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1. Introduction

Ocular onchocerciasis (river blindness) occurs primarily as a result of the host inflammatory response to dead and dying *Onchocerca volvulus* worms in the eye. In the cornea, this response is manifested first by the development of new blood vessels (neovascularization) followed by corneal opacification. Animal models have shown that development of *O. volvulus* mediated corneal inflammation (keratitis) results from the temporal recruitment of neutrophils and eosinophils to the cornea through the network of new blood vessels. The inflammatory process is dependent on development of a systemic Th2 type response to the parasite antigens [1–4]. However, we have little understanding of the mechanisms by which the parasite is capable of inducing corneal neovascularization, the seminal event in the pathologic process.

Recently the filarial genome project has begun to develop a library of expressed sequence tags from *O. volvulus* [5]. One of the most abundant cDNAs identified in infective larvae encodes proteins that demonstrate a significant degree of similarity to the vespid venom antigen 5 [6], and to the major secreted protein of *Ancylostoma caninum* infective larvae [7]. This protein was designated *Ov*-ASP-1 (for *O. volvulus* Activation associated Secreted Protein). Vespid venoms (bee and hornet venoms) are important allergens of humans. Furthermore, vespid venom antigen 5 has similarities to the testis-specific protein (Tpx) and cysteine-rich secreted protein (CRISP) family of proteins [8]. These proteins include a major autoantigen of the mammalian sperm acrosome and are found in many other vertebrate tissues [8,9].

Because the *Ov*-ASPs are similar to a component of vespid venoms and vespid venoms are capable of inducing both allergic and inflammatory responses [10], we hypothesized that these proteins may play a role in the development of ocular onchocerciasis. In the current study, we present data demonstrating that two *Ov*-ASP proteins induce an angiogenic response in naive mouse corneas, suggesting that these proteins may play a direct role in the pathogenesis of ocular onchocerciasis.

2. Materials and methods

2.1. Cloning and expression of the *Ov*-asp cDNAs

We have previously isolated a cDNA encoding one member of the *Ov*-ASP protein family by immunoscreening of an L3 cDNA expression library (OvB93-RP, GenBank Accession number AF020586), which was designated *Ov*-asp-1. The complete nucleotide sequences of two other members of the *Ov*-asp family were identified as part of the *O. volvulus* EST sequencing project and have been designated *Ov*-asp-2 (GenBank accession number H39490) and *Ov*-asp-3 (GenBank accession number AA917267). We designed three sets of primers flanking the complete open reading frames of each of the clones with *Eco*R1 sites at their 5' ends to facilitate subsequent cloning. The nucleotide sequences of the primers were as follows:

<i>Ov</i> -asp-1 <i>expf</i> :	5' GGAATTCCATATGATAC- TTTTCATCATCTTC 3'
<i>Ov</i> -asp-1 <i>expr</i> :	5' TCATTTTCTGCACAG- TCCAGA 3'
<i>Ov</i> -asp-2 <i>expf</i> :	5' GGAATTCCATATGAT- ACTTTTCTCATCTTC 3'
<i>Ov</i> -asp-2 <i>expr</i> :	5' CATAATAACTAC- TAAATATATACGT 3'
<i>Ov</i> -asp-3 <i>expf</i> :	5' GGAATTCCATATGAT- ACTTTTCATCATCTTC 3'
<i>Ov</i> -asp-3 <i>expr</i> :	5' TCATTTTTTGCA- CAATCCAGA 3'

The primer pairs were used to amplify the corresponding cDNAs from an *O. volvulus* larval cDNA library (SAW94WL-OvL3). The amplification reactions were carried out in a total volume of 200 µl in a mixture containing 20 mM Tris–HCl (pH 8.3) 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton × 100, 100 µg ml^{−1} bovine serum albumin, 200 µM dNTPs, 0.5 µM of each primer, 5 units of *Taq* DNA polymerase (Sigma Chemical, St. Louis, MO) and 2.5 units of *Pfu* DNA polymerase (Stratagene, LaJolla, CA). Cycling conditions consisted of an initial denatu-

ration of 5 min at 95°C, followed by 40 cycles consisting of 45 s at 95°C, 1 min at 50°C and 1 min at 72°C. The reaction was completed with a final step consisting of 7 min at 72°C. The reaction products were cloned into the TA-cloning vector (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Individual clones containing the PCR products were isolated, and their DNA sequence determined to ensure that the amplification process did not introduce any mutations. Inserts from clones with correct sequences corresponding to the three *Ov*-ASP proteins were excised by digestion with EcoRI and sub-cloned into the pMALCR1 expression vector (New England Biolabs, Beverly, MA). Production of recombinant maltose-binding protein (MBP) fusion proteins was induced in *Escherichia coli* using 1 mM IPTG. The fusion proteins were purified by amylose affinity chromatography as previously described [11]. Yields from the *Ov*-ASP-1/MBP and *Ov*-ASP-2/MBP preparations were in the range of 1–2 mg of protein per liter, and the purified protein was 80–90% homogeneous as judged by SDS PAGE (data not shown). Induced cultures expressing *Ov*-ASP-3/MBP did not produce stable fusion protein.

2.2. Library polymerase chain reactions

In order to investigate the expression pattern of the three *Ov*-asp transcripts, gene-specific primers were designed to amplify an approximately 400 bp fragment of the 3' region of each of the cDNA clones. Amplifications were also carried out employing primers for a cDNA encoding the glycolytic enzyme glyceraldehyde three phosphate dehydrogenase (*Ov*-gpd-1) as an internal control. The gene-specific primers used were:

Ov-asp-1f: 5'-AAACTGCTGGTACGGA-3'

Ov-asp-1r: 5'-CGAGAATTAAATGAAGAA-AAGCG-3'

Ov-asp-2f: 5'-GTTTACACCCAGCGGTCAG-ATAC-3'

Ov-asp-2r: 5'-CCAAAAATTAAATGAAGA-AATTCAATTTGC-3'

Ov-asp-3f: 5'-CGCCTTAGGGTTACCAAAA-GATG-3'

Ov-asp-3r: 5'-CAAAAATTAAATGAAGTG-AAACG-3'

Ov-gpd-1f: 5'-TGATCTCACTTGCCGACTG-C-3'

Ov-gpd-1r: 5'-AAGGTGTTGTCAGAAGGC-3'

PCR amplifications were carried out on 10 µl aliquots of each amplified *O. volvulus* cDNA library from microfilariae (mf) (SAW98MLWOvMf), second stage larvae (L2) (SAW98MLWOvL2), third stage larvae (L3) (SAW94WL-OvL3), molting third stage larvae (mL3) (SL96MLW-OvmL3), fourth stage larvae (L4) [12], adult male (SAW98MLW-OvAM) and adult female [12] using the gene-specific primer pairs. Following an initial 5 min denaturation, 35 cycles of amplification were performed using *Taq* DNA polymerase (Promega, Madison, WI) under the following cycling conditions: 95°C for 1 min, 50°C for 1 min and 72°C for 1 min. PCR products were analyzed by agarose gel electrophoresis.

2.3. DNA sequence analysis

Signal sequences and putative cleavage sites were identified using the SignalP server (<http://www.cbs.dtu.dk/services/SignalP>), employing the method of von Heijne [13]. Peptide sequences were aligned using the Gibbs sampler algorithm of MACAW program package [14]. Phylogenetic analyses were carried out on the aligned sequences using the branch and bound algorithm found in the PAUP program package [15]. The phylogenetic analysis was limited to the amino acids extending from position 24 to the end of the protein, to eliminate the putative signal sequences. Gaps introduced during the process of alignment were not considered as character states. Statistical support for the phylogeny was evaluated by bootstrap re-analysis of 1000 replicate datasets.

2.4. Assessment of angiogenic activity

The neovascular response in the corneas was

determined by slit lamp examination using the method described by Kenyon and co-workers [16]. Briefly, corneas of 4–6 week-old BALB/c mice obtained from Jackson Laboratories (Bar Harbor, ME) were injected with 30 µg of each recombinant protein in 10 µl sterile, endotoxin-free Hanks Buffered Salt Solution (Life Technologies, Gaithersburg, MD). Growth of new vessels was quantitated by measuring the vessel length from the limbus to the site of injection using a linear reticule, and by estimating the contiguous circumferential zone of neovascularization as 'clock hours' (30° of arc equals 1 h).

2.5. Histology

Eyes were removed, fixed overnight in 10% formaldehyde, and processed by standard methods and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin, and the presence of blood vessels in the corneas was determined by light microscopy.

3. Results

Full length cDNAs encoding the three members of the *Ov-asp* family were isolated by PCR amplification from a cDNA library prepared from *O. volvulus* infective larvae, as described in Materials and Methods. The derived amino acid sequences of the three members of the *Ov-ASP* family are presented in Fig. 1. The three proteins were roughly similar in predicted molecular weight (24 871 Da for *Ov-Asp-1*, 29 047 Da for *Ov-ASP-2* and 24 744 Da for *Ov-ASP-3*). All three proteins also exhibited characteristics similar to members of CRISP and Tpx protein families. The *Ov-ASPs* contain 6/10 conserved cysteine residues found in the vertebrate members of the Tpx family of proteins. All three of the members of the *Ov-ASP* family contain putative signal sequences at their amino terminal ends. In addition, the three *Ov-ASPs* contain the sequence HFTQ or a closely related variant (NFTQ), which is a conserved variation of the HYTQ sequence found in most members of the CRISP family [7]. When the sequences of the proteins minus the putative sig-

nal sequences were compared, the *Ov-ASPs* were found to be 43–58% identical and 51–66% similar to one another (Table 1). When compared to other members of the Tpx/CRISP protein family, the highest level of identity and similarity was seen between *Ov-ASP-1* and the ASP homologue of the filarial parasite *Brugia malayi*. Apart from the nematode homologues, the *Ov-ASPs* were most similar to the ASPs from *Ancylostoma caninum* and *Necator americanus* (Table 1).

When the phylogenetic relationship of the *Ov-ASP* proteins was explored, *Ov-ASP-3* and *Ov-ASP-2* formed a clade that was distinct from that formed by *Ov-ASP-1* and the ASP homologues identified in other filarial parasites (*B. malayi* and *D. immitis*), (Fig. 2). Furthermore, the filarial ASPs formed a group that was distinct from the ASP homologues of other organisms, including the ASPs from *A. caninum* and *C. elegans*. These results suggest that the filarial ASPs represent a novel subfamily of CRISP/Tpx proteins.

The discovery of three distinct *O. volvulus* clones encoding homologues of the vespid antigens suggested that the *Ov-asp* clones are members of a multigene family. To explore the structure of this family in more detail, a Southern blot prepared from *O. volvulus* genomic DNA was probed with a purified insert encoding the *Ov-asp-2* open reading frame and washed under conditions of moderate stringency (0.4 × SSC, 1% SDS at 48°C). The *Ov-asp-2* cDNA hybridized to one major band of roughly 7 kbp, and seven minor bands ranging in size from 3.5 to 16 kbp, in *HindIII* restricted *O. volvulus* DNA. In contrast, no hybridization was detected to human genomic DNA. When the blot was re-probed with a gene sequence known to be present as a single copy in the *O. volvulus* genome (the *O. volvulus* prollyl 4-hydroxylase alpha subunit I), a single distinct band was detected in the *O. volvulus* lane, confirming that the multiple bands seen were not artifacts produced either by partial digestion or degradation of the genomic DNA samples used to prepare the Southern blot (data not shown). These results, when taken together, suggest that the *Ov-asps* comprise a multi-gene family within the *O. volvulus* genome.

To gain some insight into the developmental profile of expression of the three members of the *Ov-asp* family, non-quantitative stage-specific PCR analyses were performed employing primers specific for each of the three members of the *Ov-asp* family, using cDNA libraries derived from various life stages of the parasite as template DNA. The *Ov-asp-2* transcript was found to be present in all stages, while the *Ov-asp-3* was L3 stage-specific, and the *Ov-asp-1* transcript was confined to the L2, L3, mL3 and adult female stages (Fig. 3). A search of the 4635 L3 and mL3 ESTs currently in the *O. volvulus* EST database using gene-specific segments of the three *Ov-asp* cDNAs corroborated these findings. ESTs corresponding to *Ov-asp-3* were found only in the L3 dataset, while ESTs corresponding to *Ov-asp-1* and the *Ov-asp-2* were present in both L3 and the mL3 EST datasets. Altogether, the *Ov-asp* gene cluster comprises roughly 0.81% of the L3/mL3 ESTs in the current *O. volvulus* EST database [17].

The two major clinical features of anterior ocular onchocerciasis are corneal opacification and neovascularization [1]. To determine if the *Ov-ASP*s are capable of directly inducing either of these features, *Ov-ASP-1* and *Ov-ASP-2* were expressed in *E. coli* as soluble fusion proteins with maltose binding protein (MBP) and purified to homogeneity by amylose affinity chromatography. Thirty micrograms of each purified protein were injected into the corneas of naive BALB/c mice and growth of blood vessels was monitored by slit lamp examination. As shown in Fig. 4, *Ov-ASP-2*/MBP induced a pronounced angiogenic response, with vessels growing from the peripheral cornea to the site of injection. Vessel length was maintained throughout the 7-day period, although the area of the cornea in which vessels were present (measured as clock hours) decreased after 5 days. Similar kinetics were found after injection of 10 µg protein (data not shown).

To determine the specificity of the angiogenic response, naive mice were injected with *Ov-ASP-*

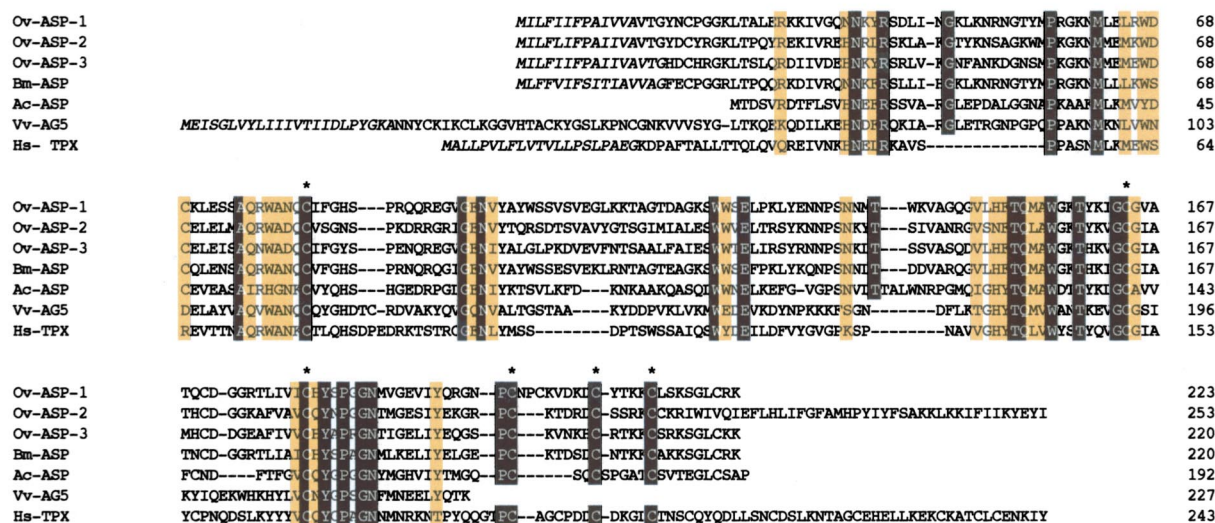


Fig. 1. Comparison of members of the *Ov-ASP* family to other members of the CRISP/Tpx family of proteins: sequences were aligned using the MACAW program as described in Section 2. Yellow shading highlights areas with high mean similarity scores, while black shading highlights identical amino acids. Italics highlight the putative signal sequences identified as described in Section 2. Asterisks highlight the conserved cysteine residues discussed in the text. *Ov-ASP-1*, *Ov-ASP-2* and *Ov-ASP-3* = members of the *Ov-ASP* family. *Bm-ASP* = *Brugia malayi* ASP homologue (accession number AF042088). *Ac-ASP* = *A. caninum* secreted protein (carboxyl terminal 192 amino acids; accession number Q16937). *Vv-Ag5* = *Vespula vulgaris* antigen 5 (accession number Q05110). *Hs-Tpx* = *Homo sapiens* Tpx protein (accession number P16562).

Table 1
Identity and similarity of the *Ov*-ASP proteins to members of the Tpx/CRISP family

Protein ^a	Identity/Similarity		
	<i>Ov</i> -ASP-1	<i>Ov</i> -ASP-2	<i>Ov</i> -ASP-3
<i>Ov</i> -ASP-2	43/51		
<i>Ov</i> -ASP-3	58/66	48/56	
<i>B. malayi</i> ASP	76/82	45/51	56/65
<i>D. immitis</i> ASP	66/73	38/46	54/62
<i>C. elegans</i> ASP	32/42	27/35	34/43
<i>A. caninum</i> ASP	33/40	27/32	32/39
<i>N. americanus</i> ASP	33/40	27/32	32/39
<i>V. vulgaris</i> Ag 5	25/31	21/26	23/31
<i>V. crabro</i> Ag 5	27/34	21/28	26/33
<i>H. sapiens</i> Tpx	20/26	24/29	25/29
<i>M. musculus</i> Tpx	20/25	20/26	24/28

^a Proteins and accession numbers are provided in the legend to Fig. 2. Amino acid groups considered similar in the similarity calculations were as follows: (I, L, V); (R, K); (N, Q); (D, E); (S, T); and (F, Y).

1/MBP, *Ov*-ASP-2/MBP, with control MBP alone, or MBP fused with the *O. volvulus* homologue of protein disulfide isomerase (*Ov*-PDI/MBP). Earlier studies have demonstrated that injection of naive mice with *Ov*-PDI/MBP did not induce keratitis [18]. However, *Ov*-PDI/MBP was extremely active in inducing keratitis in pre-immunized animals [18]. As shown in Fig. 5, angiogenesis developed in animals injected with *Ov*-ASP-1/MBP or *Ov*-ASP-2/MBP, but not in animals injected with MBP alone or *Ov*-PDI/MBP, indicating that the angiogenic response was specific for the *Ov*-ASP proteins.

To determine if the angiogenic response induced by the *Ov*-ASP/MBP proteins was associated with the presence of host inflammatory cells, eyes of mice injected with *Ov*-ASP-2/MBP or the control proteins were removed, fixed in formalin, and 5 µm paraffin sections were examined after staining with hematoxylin and eosin. Corneas of *Ov*-ASP-2/MBP — injected animals had numerous blood vessels, but relatively few inflammatory cells (Fig. 6). In contrast to the model of *O. volvulus* keratitis in which there is an intense

neutrophilic and eosinophilic infiltrate [1], most of the inflammatory cells in the cornea of *Ov*-ASP-2/MBP injected corneas were mononuclear. Similar results were seen after injection of *Ov*-ASP-1/MBP, whereas no vessels or inflammatory cells were detected in the corneas of animals injected with *Ov*-PDI/MBP or MBP alone (data not shown).

4. Discussion

One of the most abundant larval cDNAs identified by EST analysis encodes a member of the *Ov*-ASP family. The identification of three members of the *Ov*-ASP family, together with the genomic Southern blot data presented above suggest that the *Ov*-ASPs constitute a multi-gene family with several members. Furthermore, the three members of the *Ov*-ASP family characterized to date appear to exhibit different developmental profiles, suggesting that these proteins have distinct functions in the parasite. The biological function of the vespidae venom homologues in invertebrates and in *A. caninum* remains obscure. However, the *A. caninum* homologue is a major secreted protein in activated infective larvae, suggesting that this protein plays an important role in the establishment of infection [7].

Apart from the *Ov*-ASP homologues, none of the individual recombinant *O. volvulus* proteins tested to date have elicited any response in naive animals in the established mouse model for ocular onchocerciasis [18,19]. The response induced by *Ov*-ASP-1/MBP and *Ov*-ASP-2/MBP also differed significantly from that seen in animals immunized subcutaneously and challenged intrastromally with soluble native parasite antigens, a procedure that induces severe corneal opacification [3,18]. In immunized animals challenged intracorneally with total *O. volvulus* antigens, there is a pronounced migration of eosinophils and neutrophils into the cornea and severe edema. In contrast, injection of the naive animals with *Ov*-ASP-1/MBP or *Ov*-ASP-2/MBP resulted in a minimal inflammatory response. The mechanisms by which the *Ov*-ASP protein family stimulate new blood vessel formation in the

corneas of naive animals has yet to be determined. However, it is possible to envision at least two mechanisms by which this may occur. The *Ov*-ASPs may directly stimulate the growth of vascular endothelial cells to proliferate and to form new blood vessels. Alternately, angiogenesis may be as a result of the release of endothelial cell growth factors produced by infiltrating mononuclear cells. Further studies are underway to determine the relative contribution of these mechanisms.

Although corneal neovascularization is important in the development of ocular pathology associated with *O. volvulus* infection, angiogenesis may also be essential for maintaining the nodule in which the adults reside. These nodules are highly vascularized [20], and survival of these nodules may depend on production of angiogenic proteins in a manner similar to that described for tumors [21]. It may be that one function of the members of the *Ov*-ASP family is to promote neovascularization during nodule formation, thus

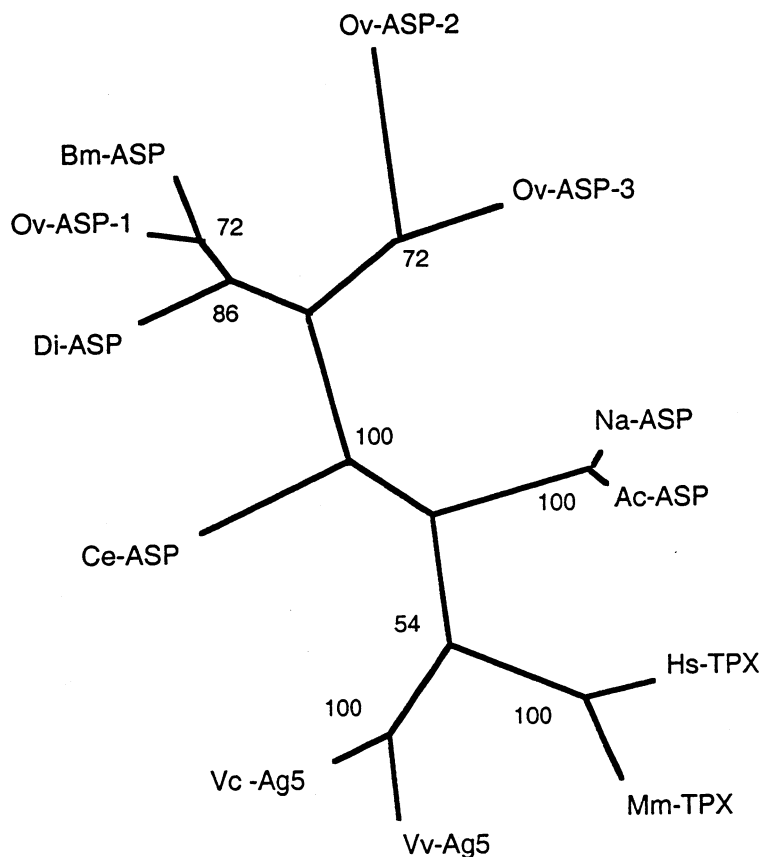


Fig. 2. Phylogenetic analysis of members of the *Ov*-ASP family: The phylogeny shown was constructed as described in Section 2. This analysis produced a single most parsimonious tree, shown here. Statistics for this phylogeny were as follows: tree length = 759 (range 678–1071); consistency index = 0.893; rescaled consistency index = 0.709. Numbers indicate bootstrap support values for the groups distal to the labeled node. Taxa are as follows: *Ov*-ASP-1, *Ov*-ASP-2, *Ov*-ASP-3 = members of the *Ov*-ASP family; *Bm*-ASP = *Brugia malayi* ASP homologue (accession number AF042088); *Di*-ASP = *Dirofilaria immitis* ASP homologue (accession number AF001100); *Ce*-ASP = *Caenorhabditis elegans* ASP homologue (accession number CAA92136); *Ac*-ASP = *Ancylostoma caninum* secreted protein (carboxyl terminal 192 amino acids, accession number Q16937); *Na*-ASP = *Necator americanus* homologue of ASP (carboxyl terminal 192 amino acids, accession number AF07952); *Vv*-Ag5 = *Vespula vulgaris* antigen 5 (accession number Q05110); *Vc*-Ag5 = *Vespa crabro* antigen 5 (accession number P35781); *Hs*-Tpx = *Homo sapiens* Tpx protein (accession number P16562); *Mm*-TPX = *Mus musculus* Tpx protein (accession number P16563).

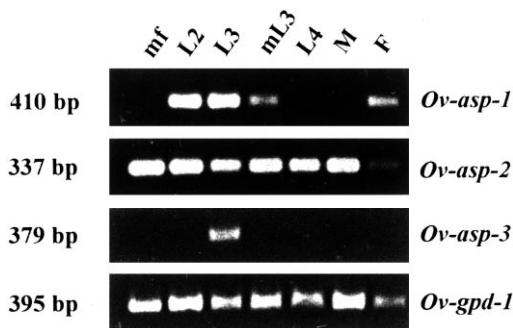


Fig. 3. Stage-specific PCR showing expression profiles of the three *O. volvulus asp* genes in different stages of the life cycle: Amplifications were carried out employing gene specific primers on cDNA libraries prepared from microfilariae (mf), second-stage larvae (L2), third-stage larvae (L3), molting larvae (mL3), fourth-stage larvae (L4), adult male and female. The *O. volvulus* gene encoding glyceraldehyde 3-phosphate dehydrogenase (*Ov-gpd-1*) was included as internal control.

ensuring a sufficient blood supply to the adult parasites. The potential importance of the members of the *Ov*-ASP family in establishing infection by the infective larvae and in the development of the nodule by the juvenile adult parasite suggests that these proteins may be a potential target for immunotherapeutic or chemotherapeutic attack against *O. volvulus* infection.

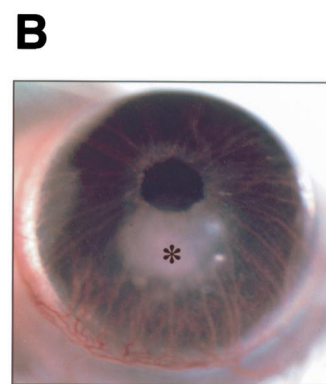
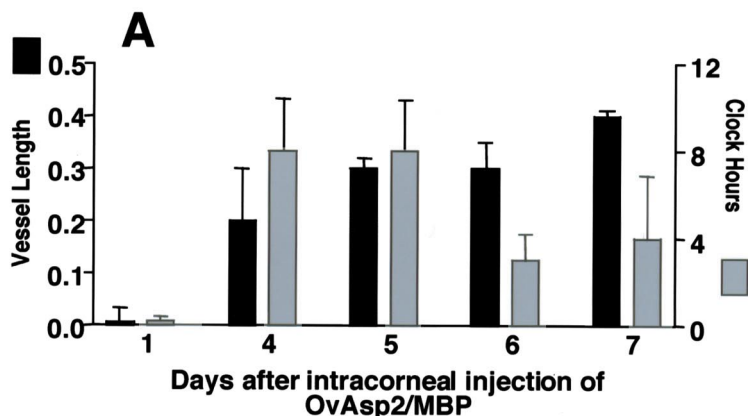


Fig. 4. Angiogenic response elicited by *Ov*-ASP-2: Panel A: 30 μ g *Ov*-ASP-2/MBP was injected into the corneal stroma of BALB/c mice. Growth of blood vessels was monitored by length (vessel length) and by circumferential area (clock hours) from the injection site over time, as described in Section 2. Data are means $\pm$ S.D. of five mice per group. This experiment was repeated five times with similar results. Panel B: photomicrograph of BALB/c mouse 7 days after injection of *Ov*-ASP-2/MBP. The asterisk indicates the site of injection.

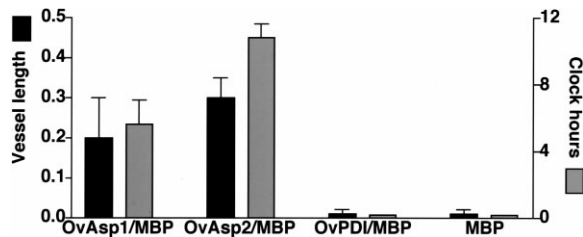


Fig. 5. Specificity of angiogenic response elicited by *Ov*-ASP/MBP fusion proteins: BALB/c mice were injected in the corneal stroma with 30 μ g *Ov*-ASP-1/MBP, *Ov*-ASP-2/MBP, *Ov*-PDI/MBP or with MBP alone. Results shown are from day 3 after injection (the midpoint of observations taken on days 1–7) and are means and S.D. of five mice per group. These data are presented as representative of what was observed at all time points between days 1 and 7. At all time points, only the *Ov*-ASP/MBP proteins induced an angiogenic response. The experiment was repeated five times with similar results.

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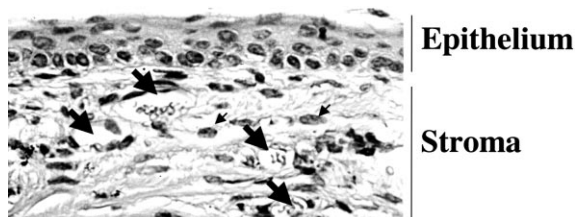


Fig. 6. Histology of mouse cornea 7 days after injection of *Ov*-ASP-2/MBP: numerous blood vessels are present in the corneal stroma (large arrows) and only occasional mononuclear cells (small arrows) are present. The micrograph is representative of five animals per group and five replicate experiments.

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