

The FAR proteins of filarial nematodes: secretion, glycosylation and lipid binding characteristics[☆]

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Abstract

The FAR proteins of nematodes are small (~20 kDa), helix-rich, fatty acid and retinol-binding (FAR) proteins that appear to be confined to nematodes. We have carried out a comparative sequence and biochemical analysis of selected FAR proteins from species of filarial parasites (from the genera *Onchocerca*, *Brugia*, *Wuchereria*, *Loa*, *Acanthocheilonema* and *Litomosoides*). The sequences fall into two main groups corresponding broadly to the onchocercal and lymphatic filariasis parasites, and only those with unshathed microfilariae were found to produce glycosylated FAR proteins. The proteins were released into culture medium by all the species and developmental stages investigated. Recombinant forms of two of these proteins (Ov-FAR-1 from *O. volvulus* and Bm-FAR-1 from *B. malayi*) were compared for ligand binding in fluorescence-based assays. Both were found to bind all-*trans*-retinol, (dansylamino) undecanoic acid (DAUDA), and oleic acid by competition. Both produced an identical, and dramatic, blue-shift in the fluorescence emission of DAUDA (from 541 to approximately 483 nm), indicative of similarity in the binding site environments of the two proteins. These findings indicate that there is strong conservation of the biochemical activities of the FAR proteins between the different parasite species, although they appear to have different post-translational modifications which may relate to the biology of the larvae. © 2002 Published by Elsevier Science B.V.

Keywords: Nematode; Glycosylation; Excretory/secretory; Fatty acid binding protein; Retinol binding protein; Filariiae

1. Introduction

Ov-FAR-1 (formerly known as Ov20) is a structurally novel small helix-rich fatty acid and retinol (Vitamin A)-binding protein produced and secreted by the filarial parasite *Onchocerca volvulus* [1,2]. Its potential role in the generation of pathology, parasite development, and survival has been discussed recently [3,4]. Ov-FAR-1 is a major antigen of *O. volvulus*, the causative agent of river blindness, which affects around 20 million people worldwide. The protein is expressed in all life-cycle stages and was shown to be secreted by adult female *O. volvulus* [1]. Homologous proteins have previously been sequenced and expressed from two other filarial para-

[☆] Note: Nucleotide sequence data reported in this paper are available in the GenBankTM, EMBL and DDBJ databases under the accession numbers: *L. loa*: AF 398373, *O. ochengi*: AY050255, *O. gutturosa*: AY050253, *O. dukei*: AY050254, *L. sigmodontis*: AY050256, *B. pahangi*: AY050257, *W. bancrofti*: AY050258. *A. viteae*.

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sites, *Brugia malayi* and *Acanthocheilonema viteae*. These recombinant proteins have been used in mouse infection models to investigate the host immune response to FAR proteins, a study not possible with *O. volvulus* [5].

The concentration of retinol within *Onchocerca* nodules is eight times higher than in surrounding host tissue [6]. Retinol sequestered from the host is thought to play an important role in normal parasite growth, differentiation and embryogenesis [7]. It is possible that the parasite, by sequestering Vitamin A, induces or exacerbates host deficiency in this vitamin. Vitamin A was first associated with *O. volvulus* infection by Rodger as early as 1957 [8], and several subsequent studies on onchocerciasis found that the vitamin A status of infected individuals has been reduced [9,10], although not all did so [11]. This may be because levels of available Vitamin A in the diet differed, with deficiency only occurring when supplies were limited. Retinoids are essential for the regulation of growth and the differentiation of skin [12–15] and this lipid is also a critical requirement for immune function, having a profound influence on both the innate and the adaptive immune responses [16]. A wide range of pathological features related to onchocerciasis, such as night blindness, destruction of the cornea, xerophthalmia and follicular hyperkeratosis, are very similar or even indistinguishable from the pathology caused by vitamin A deficiency [17]. It has, therefore, been hypothesised that retinoid binding proteins of the FAR family may play an important role in the pathological changes in the skin and eye of onchocerciasis patients, as well as in nodule formation [3]. Moreover, retinoid binding proteins of the size of the FARs have also been reported to bind ivermectin [18], which may have implications for the delivery of the drug, and/or its distribution within the parasites, leading to much higher concentrations of ivermectin within *Onchocerca* nodules compared with the surrounding host tissues [19].

Although Ov-FAR-1 may have an important role in the host-parasite interaction and the pathogenesis of onchocerciasis, we have little information on its role in nematode biology or whether its homologues may perform similar functions in other nematode species. We have, therefore, examined the FAR proteins from ten species of filarial nematodes, ranging from parasites of humans and cattle to those of laboratory models of human filariasis. The biology of these filariae differs in the habitat of adult worms (nodule-forming, non-nodule-forming) and larval stages (blood dwelling, skin dwelling, sheathed, un-sheathed microfilariae), as well as in the pathological manifestations within the host. We find that the protein sequences are closely similar, but that the glycosylation of the proteins differs between the major groups of the filariae. Moreover, we show that recombinant Bm-FAR-1 from *B. malayi* has

virtually indistinguishable lipid binding properties from recombinant Ov-FAR-1.

2. Materials and methods

2.1. cDNA cloning

Viable adult *O. gutturosa*, *O. dukei* and *O. ochengi* were collected from cattle at the abattoir in Ngaoundéré, Northern Cameroon and snap frozen in liquid nitrogen. *Litomosoides sigmodontis* was obtained from infected cotton rats and *A. vitae* was obtained from infected jirds, both of which had been maintained in the laboratory. *Brugia pahangi* was also isolated from jirds, but was supplied by Simon Townson (CAB International, St. Albans). Total RNA from all the filariae was isolated by grinding the worms to powder in liquid nitrogen, followed by total RNA isolation with TRIZOL[®] LS reagent according to the manufacturer's protocol (GIBCO BRL, Life Technologies). After treating the RNA with DNase, 2 µg was taken for the transcription to cDNA using oligo (dT) primer and M-MLV reverse transcriptase (Promega). In the case of *Wuchereria bancrofti*, a conventional adult cDNA library was provided by Steven Williams (Clark Science Center, Northampton, MA, USA).

Homologues of Ov-FAR-1 were amplified from the cDNA preparations by PCR using oligonucleotide primers that were based on the sequence of either Ov-FAR-1 or Bm-FAR-1. The homologues from *O. gutturosa*, *O. dukei*, *O. ochengi* and *L. sigmodontis* were isolated using PCR primers based on Ov-FAR-1 as previously described for *A. vitae* [5]. The forward primer (5'-CTC CAT ATG GCA AAT GTT GTT CCG TTT TC-3') was designed to generate PCR products starting from the predicted cleavage site (Asn 17) of the secretory signal peptide of Ov-FAR-1 and the reverse primer (5'-CTC GGA TCC TTA ATG TTT TCC GGC ACC-3') was based on the 3'-UTR of the Ov-FAR-1 gene. Fragments of the Bp-FAR-1 and the Wb-FAR-1 genes were isolated using PCR primers based on the sequence of Bm-FAR-1. The forward primer was 5'-CGA TTG ATT CTG CTG GCA-3' and the reverse primer was 5'-GGT ACT CGC GTT GCT TTT GA-3'. PCR products of the expected size were gel purified (Sepaglas[™] Band Prep Kit, Pharmacia Biotech) and cloned into the TOPO TA cloning plasmid (Invitrogen). Template plasmid for sequencing was prepared according to the Wizard Miniprep kit protocol (Promega) and sent for commercial automatic sequencing (Cambridge, Biosciences and MWG-Biotech Ltd).

A variety of strategies were subsequently employed to isolate and sequence the missing 5'- and 3'-ends of the open reading frames of the newly cloned FAR genes. The 5'-end of Od-FAR-1 was amplified by PCR using

an oligonucleotide forward primer (5'-GGT TTA ATT ACC CAA GTT TGA G-3') based on the SL1 spliced-leader sequence and a reverse primer (5'-ATA TTT AGC AAT GAT GTC TCG A-3') based on an internal sequence in the Od-FAR-1 gene. The PCR product was then cloned and sequenced as described above. As a similar approach was unsuccessful with the other filarial homologues of Ov-FAR-1, a new forward PCR primer (5'-CAG TCA AGA AAC TCT CGT GGA-3') based on conserved nucleotides in the 5'-UTR of Od-FAR-1 and Ov-FAR-1 and a reverse primer (5'-AAC GAT CGT ACC TTG GCA AT-3') based on an internal sequence in Og-FAR-1, were used to amplify the 5'-ends of the Og-FAR-1, Oo-FAR-1, Ls-FAR-1 and Av-FAR-1 sequences. The 5'-end of the open reading frame of the Bp-FAR-1 and the Wb-FAR-1 genes was isolated using a forward PCR primer (5'-GAG AAA CTT TCG TTG ACA ATT A-3') based on the 5'-UTR of Bm-FAR-1 and a reverse primer (5'-AAT ATT TTA GCA TCT GGC TTT-3') based on a sequence within the open reading frame of Bm-FAR-1 that is conserved in Bp-FAR-1 and Wb-FAR-1. The 3'-end of the open reading frame of Bp-FAR-1 and Wb-FAR-1 was isolated using a forward PCR primer (5'-ATG AAG ATG CTG CAC TGG AA-3') based on a conserved oligonucleotide sequence in the open reading frame of the Bm-FAR-1, Bp-FAR-1 and Wb-FAR-1 genes, and a reverse PCR primer based on the 3'-UTR of Bm-FAR-1 (5'-GTT TCG ATG GCC AAA CTT CT-3'). PCR products of the expected size were cloned and sequenced as described above.

For the identification of the *Loa loa* homologue, a partial sequence was obtained during sequencing of inserts produced by PCR amplification of random plaques from a conventional *L. loa* L3 cDNA library using commercially available M13F and M13R primers (Stratagene). An overlapping partial cDNA sequence was identified using the following primers: 5' AGG TCT GAC GGC TGA AGA TAA 3' and M13F.

The filarial FAR sequences were analysed with the BLAST programs available online at <http://www.ncbi.nlm.nih.gov/BLAST>. Alignment of the amino acid sequences was done using Clustal W online at <http://www.ebi.ac.uk/index.html>. Phylogenetic analysis was carried out using maximum likelihood [20] protein sequence parsimony method and the unweighted pair group method with arithmetic mean [21]. The trees were rooted with the As-FAR-1 sequence of *Ascaris suum* as an outgroup, this nematode being a member of the same major clade of nematodes as the filariae, but with a FAR protein sequence significantly different from that of the filariae. All methods produced trees of the same topology and a typical result with bootstrap values is presented. The sequence information of the seven new FAR binding protein homologues of *O. gutturosa*, *O. dukei*, *O. ochengi*, *L. sigmodontis*, *B. pahangi*, *W.*

bancrofti and *L. loa*, were analysed together with the previously published homologues of *O. volvulus* [22], accession number L27686, and *B. malayi* [5], accession number U69169.

The nomenclature of the FAR proteins is as proposed by Bradley et al. [4], and follows the convention that the first two letters of the name come from the genus and species. As yet, only one sequence has been found in any species of filarial nematode, but, in anticipation of there being more (as have been found in *Caenorhabditis elegans*) the FAR proteins and their encoding proteins have been given the suffix '-1'. Thus, the FAR of *B. malayi* is Bm-far-1 (gene) and Bm-FAR-1 (protein).

2.2. Fluorescence-based lipid binding assays

cDNA encoding Bm-FAR-1 and Ov-FAR-1, without their hydrophobic/signal sequences, was subcloned into the expression vector pET-15b (Novagen), and recombinant protein expressed in *E. coli* BL21(DE3), and affinity purified as described by Kennedy et al. [2] and Nirmalan et al. [5], respectively. The recombinant proteins were passed down ExtractiGel D columns (Pierce) to remove any contaminating detergent, and this procedure probably also removes any lipids. The concentrations of the proteins were estimated by absorbance at 280 nm, using extinction coefficients based on their amino acid compositions, and calculated using the ProtParam tool online at <http://ca.expasy.org/tools/protparam.html>. Lipid binding was detected spectrofluorometrically, using the fluorescent fatty acid analogue (dansylamino)undecanoic acid (DAUDA), which bears the environment-sensitive dansyl fluorophore, and all-*trans*-retinol, as described previously [2]. The fluorescence spectra are uncorrected and were analysed using MICROCAL ORIGIN software.

2.3. Excretion/secretion (EIS) of FAR proteins

Adult filariae (*O. volvulus*, *O. ochengi*, *O. gutturosa*, *A. viteae*, *L. sigmodontis*) and microfilariae (*O. ochengi*, *L. sigmodontis*) were metabolically labelled for 24 h with 5.3 MBq ml⁻¹ (³⁵S)methionine in RPMI 1640 (0.2 mg ml⁻¹ gentamycin) after a starvation period of 4 h in methionine free medium. The culture supernatant was 6 × concentrated in 3 kDa cut off spin columns (NanosepTM, Pale Filtron Corporation) and immunoprecipitated with a polyclonal mouse antiserum raised in BALB/c mice against a recombinant Ov-FAR-1 protein [1]. The precipitate was then re-suspended in loading buffer for SDS-PAGE, fractionated by SDS-Tricine polyacrylamide gel electrophoresis [23] and the radiolabelled proteins detected by autoradiography. Culture supernatant of *O. ochengi* microfilariae that had been purified by gel filtration using a Sephadex PD10 chromatography column as previously described [24]

was also used for Western Blotting analysis and probed with a polyclonal rabbit antiserum against recombinant Ov-FAR-1. The supernatant was freeze-dried and diluted in 1/50 of the original volume in SDS loading buffer before separation by SDS–Tricine polyacrylamide gel electrophoresis. Polyclonal antiserum against the recombinant Ov-FAR-1 His₆ tag protein was raised in a rabbit by injecting 2 × 100 µl of 100 µg ml⁻¹ Ov-FAR-1 His₆ and boosted with the same amount after 21 and 42 days in the MPL-TDM adjuvant system according to the manufacturer's recommendations (Sigma M6536). Titre and specificity were tested by ELISA and sera collected after the third boost.

2.4. Glycosylation of parasite-derived FAR proteins

In order to analyse whether or not the predicted glycosylation sites in the FAR amino acid sequences were actually glycosylated *in vivo*, parasite extracts were treated with PNGase F (New England Biolabs). Somatic antigens of the different species were prepared by homogenising the parasites in phosphate buffered saline on ice, subsequent sonication, ultra-centrifugation and filtration according to [25]. Adult male and female parasites were used, except for *L. loa*, where the extract was made up from infective third stage larvae. For the deglycosylation reactions 10 µg of each parasite extracts were used according to the manufacturer's protocol. As controls 10 µg of parasite extracts were taken up into the same buffers as for the digestion reaction, with the exclusion of the enzyme. Undigested and digested extract were subsequently separated by SDS-PAGE, blotted and probed with anti-Ov-FAR-1 rabbit serum.

3. Results

3.1. Amino acid sequence comparison

cDNA encoding homologues of Ov-FAR-1 were cloned from *O. gutturosa*, *O. dukei*, *O. ochengi*, *L. sigmodontis*, *B. pahangi*, *W. bancrofti* and *L. loa* and compared with the previously published sequences from *O. volvulus* [22], *A. viteae* and *B. malayi* [5]. The protein sequences were closely similar or identical (Fig. 1), with identities ranging from 100% between Ov-FAR-1, Oo-FAR-1 and Ls-FAR-1, to 79% between LI-FAR-1 and Av-FAR-1 (Table 1). A distance-based analysis of the FAR proteins is presented in Fig. 2, showing two main clusters (*Onchocerca/Acanthocheilonema/Litomosoides* and *Brugia/Wuchereria/Loa*).

The FAR proteins of the four *Onchocerca* species, *L. sigmodontis* and *A. viteae* have potential N-linked glycosylation sites at three identical positions in the amino acid sequence (Fig. 1). None of these potential N-linked glycosylation sites are present in the *B. pahangi*,

B. malayi, *W. bancrofti* or *L. loa* proteins. Bm-FAR-1 has a single consensus N-linked glycosylation site near the carboxy terminus of the protein (amino acid position 170–172) and LI-FAR-1 has a single site predicted to occur towards the amino terminus (amino acid position 24–26). Bp-FAR-1 and Wb-FAR-1, however, do not contain any putative N-linked glycosylation sites.

Secondary structure sequence-based predictions using the GOR, PredictProtein and Jpred suites of programs available through the Expasy server (<http://ca.expasy.org/tools/>) indicated that all of the proteins are helix-rich, with no prediction for beta/extended structure. For Ov-FAR-1 [2] and members of the FAR family from *C. elegans* (A. Garofalo et al. unpublished results), this prediction has been confirmed by circular dichroism. It thus appears to be a universal feature of the FAR proteins and discriminates them from mammalian lipid binding proteins of similar size in vertebrates (such as the ~20 kDa lipocalins, and the ~14 kDa members of the FABP/P2/CRBP/CRABP family). An interesting feature first noticed with Ov-FAR-1 is the high probability predictions for two distinct regions of coiled coil between positions 61 and 89 and 119 and 155 (probabilities 0.78 and 1.00, respectively). Bm-FAR-1 also appears to have two distinct regions of coiled coil between positions 61 and 89 and 123 and 154 (probabilities 0.84 and 0.99, respectively). Within the FAR family of filarial nematodes, the strength of the coiled-coil prediction varies, but appears to be a consistent feature.

3.2. N-linked glycosylation patterns

As described above, there were three predicted glycosylation sites in the Ov-FAR-1 homologues of *O. ochengi*, *O. dukei*, *O. gutturosa* and *O. volvulus*, as well as in *L. sigmodontis* and *A. viteae*. In order to investigate the size of the native proteins relative to their predicted size based on amino acid sequence, an immunoprecipitation was performed using labelled E/S and a polyclonal antisera to Ov-FAR-1 protein. These experiments revealed that the native FAR proteins of *Onchocerca* species were 20 and 22 kDa (Fig. 3A), including an additional 17 kDa precipitated band in the *O. ochengi* extract. The native FAR protein of *L. sigmodontis* migrated at 17 kDa and that of *A. viteae* at 20 kDa (Fig. 3B).

Sequence analysis revealed no N-linked glycosylation sites for either Bp-FAR-1 or Wb-FAR-1 and only one predicted glycosylation site each for Bm-FAR-1 and LI-FAR-1. The position of the N-linked glycosylation sites in Bm-FAR-1 and LI-FAR-1 was distinct from any of the three sites in the FAR proteins of *Onchocerca* species. The native FAR proteins of *B. pahangi*, *W. bancrofti* and *L. loa* migrated at around 17 kDa (Fig. 4). Treatment of the native FAR proteins from the different

	1								60
Ov-FAR-1	myhqlilmal	igvimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u NLTQEDR	QILRELASKH			
Od-FAR-1	myhqlilmal	igvimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u NLTQEDR	QILRELASKH			
Og-FAR-1	myhqlilmal	igvimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u NLTQEDR	QILRELASKH			
Oo-FAR-1	myhqlilmal	igvimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u NLTQEDR	QILRELASKH			
Av-FAR-1	myhqlilmal	igvimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u NLTQEDR	QILRELASKH			
Ls-FAR-1	myhqlilmal	igvimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u NLTQEDR	QILRELASKH			
Ll-FAR-1	myhqlilmal	igtimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u LTQEDR	QILRELASKH			
Bm-FAR-1	myhqlilmal	igtimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u LTQEDR	QILRELASKH			
Bp-FAR-1	myhqlilmal	igtimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u LTQEDR	QILRELASKH			
Wb-FAR-1	myhqlilmal	igtimaNVVP	FMSNIP ^u EEY	KEFIPEEVKN	FYK ^u LTQEDR	QILRELASKH			
	61								120
Ov-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Od-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Og-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Oo-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Av-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Ls-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Ll-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Bm-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Bp-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
Wb-FAR-1	ATFTNEDAAL	EALKNKSDKL	YQKAVELRNF	VKAKIDSLKP	DAKAFVDEII	AKVRSR ^u RPED			
	121								178
Ov-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Od-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETREE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Og-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Oo-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Av-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Ls-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Ll-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Bm-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Bp-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			
Wb-FAR-1	GQKLDMEK ^u LK	QAARDIIAKY	EALNEETKEE	LKATFPNTTK	IITNEKFKRI	ANSFLQKN			

Fig. 1. Amino acid sequence alignments of the FAR proteins of different filarial parasites. Potential N-linked glycosylation sites are underscored and those amino acids that are not conserved at a particular position compared with the sequence of Ov-FAR-1 are shaded in grey.

species with PNGase F to remove N-linked carbohydrate residues from the protein, produced 17 kDa non-glycosylated entities in all species (Fig. 4), which is close to the molecular mass of ~ 18 kDa predicted from their amino acid compositions. We concluded that Ls-FAR-

1, Bp-FAR-1 and Ll-FAR-1 were not glycosylated, whereas Oo-FAR-1, Od-FAR-1, and Av-FAR-1 were, although the possibility of small O-glycosylations cannot be excluded for any. In the *Onchocerca* species the FAR proteins were present in two different degrees of

Table 1
Sequence similarities between the FAR proteins

	Ov	Od	Og	Oo	Av	Ls	Ll	Bm	Bp
Wb	82 (147)	82 (146)	83 (149)	82 (147)	81 (145)	82 (147)	90 (161)	98 (175)	98 (175)
Bp	81 (145)	80 (144)	82 (146)	81 (145)	80 (143)	81 (145)	89 (159)	98 (176)	
Bm	81 (145)	80 (144)	82 (146)	81 (145)	80 (143)	81 (145)	89 (160)		
Ll	80 (144)	80 (143)	80 (144)	80 (144)	79 (142)	80 (144)			
Ls	100 (178)	98 (175)	98 (176)	100 (178)	98 (175)				
Av	98 (175)	96 (172)	97 (173)	98 (175)					
Oo	100 (178)	98 (175)	98 (176)						
Og	98 (176)	97 (173)							
Od	98 (175)								

The values in bold typeface give the percent of identical amino acid positions in each alignment and the values in brackets give the total number of identical amino acids in each alignment.

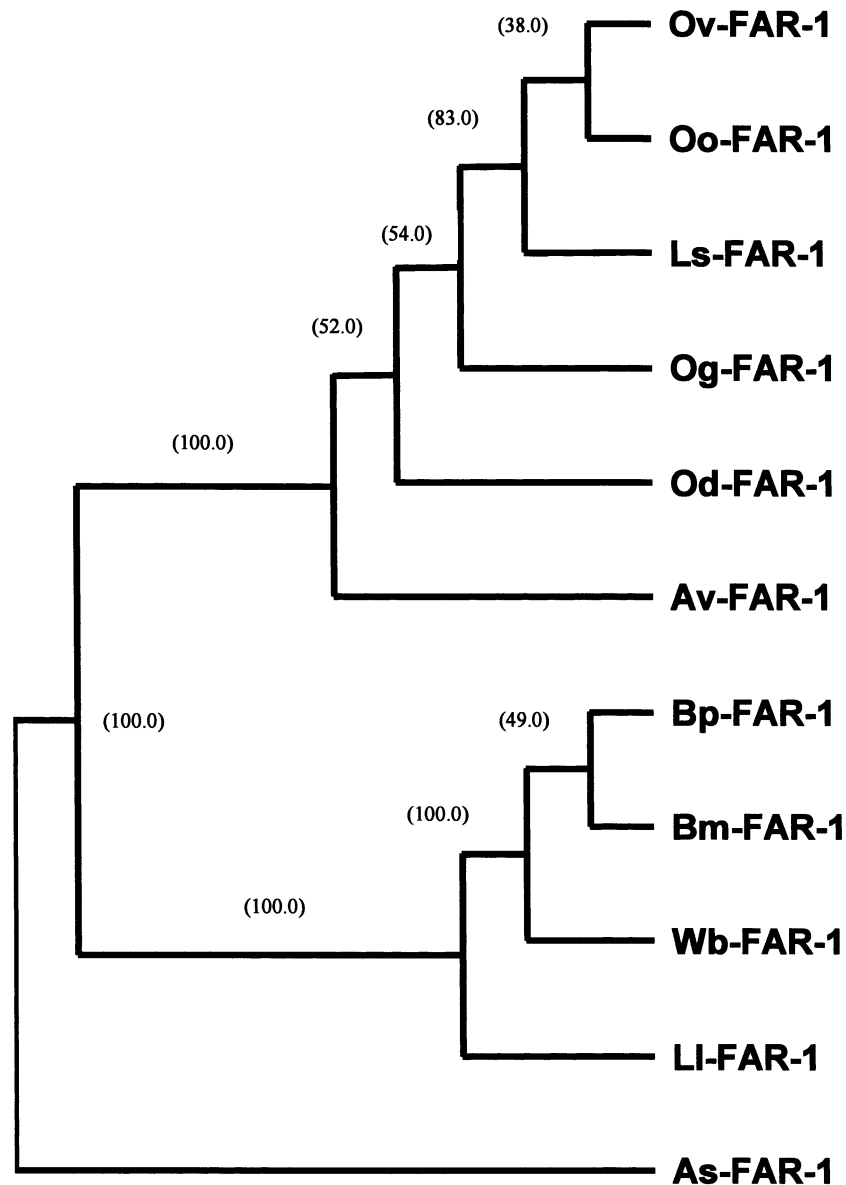


Fig. 2. Phylogenetic relationships between the predicted amino acid sequence of As-FAR-1 and the filarial FAR proteins. The bootstrap values are shown in parenthesis.

glycosylation, leading to a 20 and a 22 kDa native protein, whereas in *A. viteae* only one form of 20 kDa native protein was present (Table 2). This suggests that a proportion of the proteins utilise two glycosylation sites and some use only one. Furthermore, the experiment also verified the expression of the FAR protein in third stage larvae, as the extract used for *L. loa* was derived from infective stage larvae, instead of from adult stages.

3.3. Excretion/secretion of FAR proteins

The immuno-precipitation experiment described above using metabolically labelled E/S products also provided direct evidence that the FAR proteins are secreted (Fig. 3A and B). Importantly, these studies also

showed that the blood-dwelling microfilariae of *L. sigmodontis* (immunoprecipitation, Fig. 3B), as well as the skin dwelling microfilariae of *O. ochengi* (immunoprecipitation not shown; and Western blot analysis, Fig. 3C) released FAR protein into their surroundings. In the case of *L. sigmodontis* and *A. viteae*, it could be shown that both male and female adults released the protein, and that glycosylation patterns were the same between the sexes.

3.4. Lipid binding

The fatty acid and retinol binding properties of Ov-FAR-1 have already been described in detail [2], and here we selected one of the new FAR proteins, Bm-

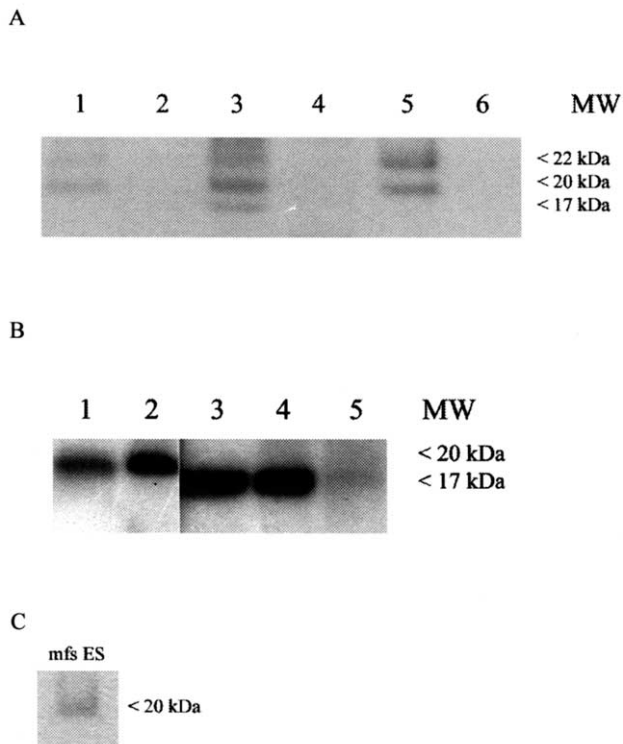


Fig. 3. FAR proteins in the culture supernatant (SN): (A) Immunoprecipitation with polyclonal mouse anti-Ov-FAR-1 serum: *O. volvulus* (lane 1), *O. ochengi* (lane 3), *O. gutturosa* (lane 5). Lanes 2, 4, and 6 were with pre-immune sera for each of the respective parasites. (B) Immunoprecipitation with polyclonal rabbit anti-Ov-FAR-1 serum: *A. viteae* (lane 1: adult male SN, lane 2: adult female SN) and *L. sigmodontis* (lane 3: adult male SN, lane 4: adult female SN, lane 5: microfilariae SN). (C) Western blot analysis of *O. ochengi* microfilariae SN using polyclonal rabbit anti-Ov-FAR-1 serum.

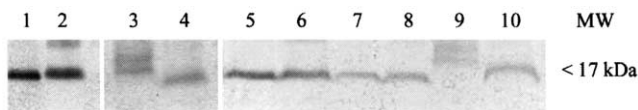


Fig. 4. Identification of native FAR proteins by immunoblot analysis of worm extracts before and after deglycosylation with PNGaseF using *L. loa* (lane 1 before, lane 2 after digestion), *O. dukei* (lane 3 before, 4 after digestion), *L. sigmodontis* (lane 5 before, 6 after digestion) *B. pahangi* (lane 7 before, 8 after digestion) and *O. ochengi* (lane 9 before, 10 after).

FAR-1, for comparative analysis. This protein was selected because it comes from a parasite whose mammalian stages have different tissue environments from *O. volvulus*, the microfilariae are sheathed, and the natural FAR protein is non-glycosylated. The fluorescence of retinol upon mixing with Bm-FAR-1 underwent a dramatic increase, indicative of entry into a protein binding site. This enhancement of fluorescence emission was partially reversed upon addition of oleic acid, indicating congruence, or interaction, between the binding sites for the two lipids (Fig. 5). Perhaps more informatively, Bm-FAR-1 and Ov-FAR-1 exhibited

very similar binding characteristics with DAUDA, a fluorescent fatty acid analogue whose emission characteristics are environment-sensitive. Fig. 6A shows that the proteins both produced substantial increases in DAUDA fluorescence, but, more informatively, they produced identical blue-shifts in the wavelength of peak fluorescence emission (from 542 to ~483 nm). The degree of blue shift in emission by the dansyl fluorophore is taken as being indicative of the degree of apolarity of a protein's binding site and isolation from solvent water. A shift to ~483 nm is greater than that observed with vertebrate lipid binding proteins, and equivalent to that found with the polyprotein allergen proteins of nematodes [26], which are also highly helical but unrelated to the FAR proteins. As with retinol, oleic acid competed effectively with DAUDA for binding (Fig. 6B for Bm-FAR-1). Our previous analysis of Ov-FAR-1 relied on recombinant protein that would not have been glycosylated and the fact that its binding properties are virtually indistinguishable from those of Bm-FAR-1 (which is not glycosylated by the worm) indicates that the recombinant proteins fold to form very similar lipid binding sites, but it remains to be seen whether glycosylation alters the lipid binding characteristics of these proteins.

4. Discussion

The purpose of this study was to compare the sequence similarities, ligand binding, and glycosylation patterns of the FAR proteins of filarial parasites with the biological characteristics of each species. One hypothesis was that glycosylation or excretion of the different FAR proteins could be correlated with lipid binding capacity, and, therefore, relate to skin pathology and nodule formation of *Onchocerca* sp. However, we found no essential differences between the excretion of the homologues by parasites in vitro or the lipid binding characteristics of recombinant Bm-FAR-1 (from a lymphatic species) and Ov-FAR-1 (from a nodule forming species). Although the amino acid sequences of the filarial FAR proteins are very similar, differences were evident in the location and number of N-linked glycosylation sites, and the utilisation of these sites. The native FAR proteins of the nodule-forming, subcutaneously-situated filariae, *O. volvulus* and *O. dukei*, occurred as glycosylated molecules of 20 and 22 kDa, whereas a substantial proportion of the native FAR proteins of *O. ochengi* and *O. gutturosa* were secreted in their non-glycosylated form at 17 kDa, in addition to the 20 and 22 kDa forms. In contrast to *O. volvulus* and *O. dukei*, *O. ochengi* is intradermally situated, surrounded by only a very thin nodule wall, and *O. gutturosa* is situated free in the connective tissue of the ligamentum nuchae. The heterogeneity of mole-

Table 2
Relationship between parasitological parameters and FAR protein glycosylation patterns

Species	Adult niche	Mf niche	Mf sheath	Glycosylation	MW (kDa) of the native FAR
<i>O. volvulus</i>	Nodule	Cuticular	No	Yes	20,22
<i>O. ochengi</i>	Nodule	Cuticular	No	Yes	17,20,22
<i>O. dukei</i>	Nodule	Cuticular	No	Yes	20,22
<i>O. gutturosa</i>	Connective tissue	Cuticular	No	Yes	17,20,22
<i>A. vitea</i>	Subcuticular	Blood	No	Yes	20
<i>L. sigmodontis</i>	Pleura	Blood	Yes	No	17
<i>B. pahangi</i>	Lymphatics	Blood	Yes	No	17
<i>B. malayi</i>	Lymphatics	Blood	Yes	No	17
<i>W. bancrofti</i>	Lymphatics	Blood	Yes	n.d.*	n.d.*
<i>L. loa</i>	Connective tissue	Blood	Yes	No	17

not determined*.

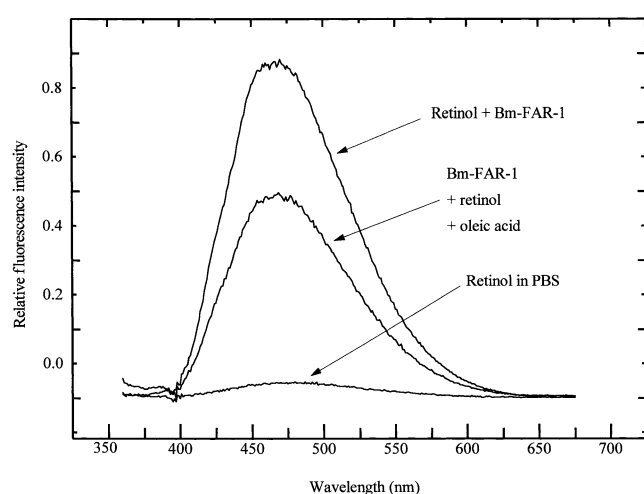


Fig. 5. Retinol binding by the recombinant FAR protein of *B. malayi* (Bm-FAR-1) and competition for binding by oleic acid. Retinol (5 μ l of 188 μ M retinol in ethanol) was added to 2 ml of phosphate buffered saline pH 7.3 (PBS) or 2 ml PBS containing 1 μ M recombinant Bm-FAR-1. To the latter was then added 10 μ l of a 1.06 mM solution of oleic acid in PBS and a new emission spectrum was recorded. λ_{exc} = 350 nm; Raman spectrum of the buffer subtracted.

cular mass of the native *Onchocerca* FAR proteins in immunoprecipitation and immuno blot analysis showing a 20 and a 22 kDa molecule, are likely due to different glycosylation i.e. a glycosylation site is used or not, or that the antennae may differ dramatically. The FAR protein of *A. viteae* was glycosylated and occurred exclusively as a 20 kDa molecule. Although *L. sigmodontis* has the same three glycosylation sites and appears to cluster with the *Onchocerca* species and *A. viteae* which are all glycosylated, its FAR protein was not. The FAR proteins of *B. malayi* and *L. loa* contain a single glycosylation site, but they are not utilised. The only feature that correlated with glycosylation of FAR proteins (but not potential number of glycosylation sites) was the absence of a microfilarial sheath, such that species possessing a sheath have non-glycosylated FAR proteins (Table 2). The absence of glycosylation in sheathed filarial species might be relevant to the transfer

of FAR proteins through the sheath but the biological significance of these glycosylation differences remain to be established.

FAR proteins are more likely to have a role in the pathogenesis of disease or interaction with the host tissues if they are secreted. Whilst Ov-FAR-1 has previously been found in the E/S of adult female worms by immunoblotting [1], it was unclear whether the release occurs naturally in vivo given that its presence in in vitro culture medium could be ascribed instead to antigen released from damaged or dead worms. We therefore undertook metabolic labelling experiments and found FAR proteins in the supernatant of cultures of adult male and female parasites of viable *A. viteae*, *L. sigmodontis*, *O. ochengi*, *O. gutturosa* and *O. volvulus*, Microfilariae of *L. sigmodontis* (sheathed) and microfilariae of *O. ochengi* (unsheathed) also both released FAR protein into culture medium. In addition, the expression of the FAR protein was confirmed in third stage larvae of *L. loa*. It appeared that all filarial parasites secrete the FAR proteins regardless of glycosylation status or differences in the biology of the parasite.

The comparative sequence analysis of the FAR homologues often filarial parasites demonstrated that they are very closely related, conserved proteins. As yet, however, we do not understand their role in the biology of the parasite or whether they are important in the host-parasite relationship. By analogy with other organisms [27], parasitic nematodes probably require retinol for a variety of their metabolic and developmental demands, such as growth, differentiation, embryogenesis, glycoprotein synthesis, and as anti-oxidants. Some of these requirements have been demonstrated in vitro, and in vivo, where retarded development of mf in the uteri of *L. sigmodontis* occurred in cotton rats, depleted of Vitamin A [7]. Experiments with radiolabelled retinoic acid in *B. malayi*, showed that it is taken up by worms and localized at high concentrations in early and late embryonic forms, further suggesting an important role for retinoids in growth and development

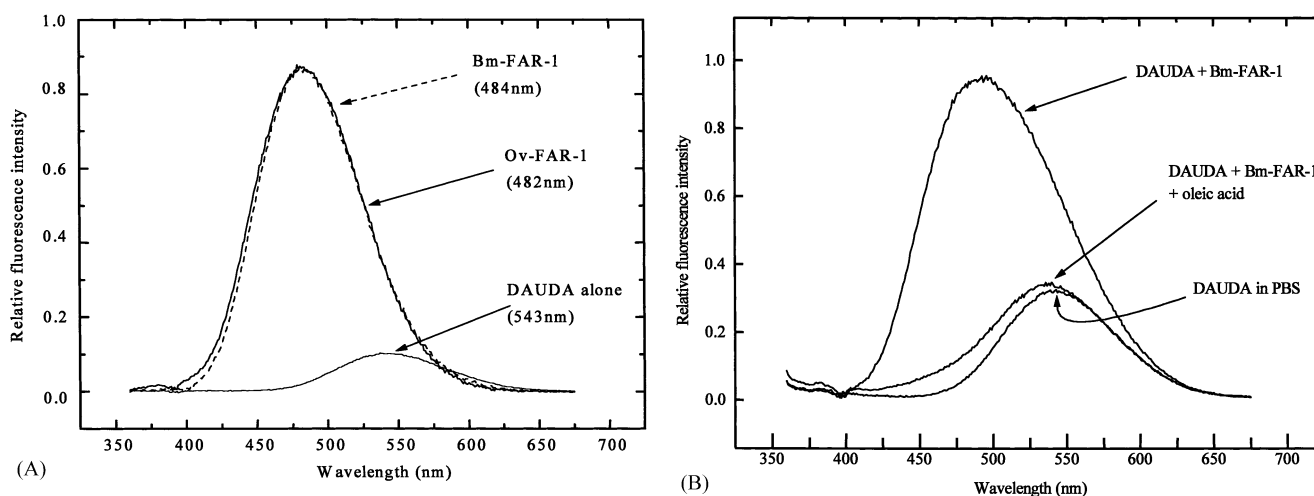


Fig. 6. Comparative DAUDA binding properties of Bm-FAR-1 and Ov-FAR-1. (A) Bm-FAR-1 (final concentration 1 μ M) and Ov-FAR-1 (final concentration 8.2 μ M) added to 2 ml of an approximately 1 μ M solution of DAUDA in PBS. The fluorescence emission spectra have been rescaled better to illustrate the similar change in DAUDA emission characteristics upon addition of either of the two proteins. The spectra of the samples with protein added have been corrected by subtraction from the spectrum of DAUDA in buffer alone to show the emission of DAUDA when in the protein binding site. (B) The effect of addition of 10 μ l of a 1.06 mM solution of oleic acid (final concentration 5.2 μ M) to a pre-formed mixture of Bm-FAR-1 and 1 μ M DAUDA. λ_{exc} = 345 nm; Raman corrected.

[28]. Studies on filarial parasites *in vitro* have also demonstrated that synthetic retinoids can reduce motility, inhibit release of microfilariae [29] and inhibit moulting of the larval stages [30]. Studies on the requirements for retinol and the role of the FARs as retinoid binding proteins in the biology of filarial parasites are difficult due to their complex life cycles, but the free-living nematode *C. elegans* has eight homologues [4]. It is therefore possible that this extremely well-characterised system can be used as a model to investigate their function by reverse genetics and functional genomics.

Whether or not the release of the parasitic FAR proteins into the surrounding host tissues (either by the adult worms or the larval stages) contributes towards the pathological changes in skin, lymphatics or eye, has yet to be seen. Since the recombinant FAR proteins have been produced in *Escherichia coli* in their non-glycosylated forms, it will be important to establish how native proteins behave in binding studies once sufficient quantities become available. At this point it can only be speculated whether the release of the FAR proteins and their high affinity for Vitamin A may cause localised vitamin depletion. This effect which would not be detectable by quantifying Vitamin A levels in serum, might result in impairment of local immune responses and, therefore, lead to pathological changes. The relationships between parasite FAR proteins, host immune responses, and pathogenesis clearly merit further investigation.

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References

- [1] Tree T.I., Gillespie A.J., Shepley K.J., Blaxter M.L., Tuan R.S., Bradley J.E.. Characterisation of an immunodominant glycoprotein antigen of *Onchocerca volvulus* with homologues in other filarial nematodes and *Caenorhabditis elegans*. *Mol Biochem Parasitol* 1995;69:185–95.
- [2] Kennedy M.W., Garside L.H., Goodrick L.E., McDermott L., Brass A., Price N.C., Kelly S.M., Cooper A., Bradley J.E.. The Ov20 protein of the parasitic nematode *Onchocerca volvulus*. A structurally novel class of small helix-rich retinol-binding proteins. *J Biol Chem* 1997;272:29442–8.
- [3] Bradley J., Kennedy M.. Retinoids, novel retinoid-binding proteins and river blindness. *Retinoids* 1999;15:11–4.
- [4] Bradley J.E., Nirmalan N., Klager S.L., Faulkner H., Kennedy M.W.. River blindness: a role for parasite retinoid-binding

- proteins in the generation of pathology. *Trends Parasitol* 2001;17:471–5.
- [5] Nirmalan N., Cordeiro N.J., Klager S.L., Bradley J.E., Alien J.E.. Comparative analysis of glycosylated and nonglycosylated filarial homologues of the 20-kilodalton retinol binding protein from *Onchocerca volvulus* (Ov20). *Infect Immun* 1999;67:6329–34.
- [6] Sturchler D., Wyss F., Hanck A.. Retinol, onchocerciasis and *Onchocerca volvulus*. *Trans R Soc Trop Med Hyg* 1981;75:617.
- [7] Storey D.M.. Vitamin A deficiency and the development of *Litomosoides carinii* (Nematoda, Filarioidea) in cotton rats. *Z Parasitenkd* 1982;67:309–15.
- [8] Rodger F.. New observations on ocular onchocerciasis, related pathological methods and the pathogenesis of various eye lesions. *Bull World Health Organisation* 1957;16:495.
- [9] Haller L., Lauber E.. Interactions between blood levels of vitamins and parasitic diseases common in the tropical zone. *Acta Trop* 1980;37:110–9.
- [10] Mustafa K.Y., Turunen U., Gumaa K.A.. Serum vitamin A levels of patients with onchocerciasis from two areas of the Sudan. *J Trop Med Hyg* 1979;82:122–7.
- [11] Williams J.F., Abu Yousif A.H., Ballard M., Awad R., el Tayeb M., Rasheed M.. Onchocerciasis in Sudan: the Abu Homed focus. *Trans R Soc Trop Med Hyg* 1985;79:464–8.
- [12] Benya P.D., Padilla S.R.. Modulation of the rabbit chondrocyte phenotype by retinoic acid terminates type II collagen synthesis without inducing type I collagen: the modulated phenotype differs from that produced by subculture. *Dev Biol* 1986;118:296–305.
- [13] Chen M., Goyal S., Cai X., O'Toole E.A., Woodley D.T.. Modulation of type VII collagen (anchoring fibril) expression by retinoids in human skin cells. *Biochim Biophys Acta* 1997;1351:333–40.
- [14] Nelson D.L., Balian G.. The effect of retinoic acid on collagen synthesis by human dermal fibroblasts. *Coll Relat Res* 1984;4:119–28.
- [15] Varani J., Mitra R.S., Gibbs D., Phan S.H., Dixit V.M., Mitra R., Jr, Wang T., Siebert K.J., Nickoloff B.J., Voorhees J.J.. All-*trans* retinoic acid stimulates growth and extracellular matrix production in growth-inhibited cultured human skin fibroblasts. *J Invest Dermatol* 1990;94:717–23.
- [16] Stephensen C.B.. Vitamin A, infection, and immune function. *Annu Rev Nutr* 2001;21:167–92.
- [17] Thylefors B.. Prevention of blindness: the current focus. *WHO Chron* 1985;39:149–54.
- [18] Lal P.G., James E.R.. *Onchocerca* retinol- and ivermectin-binding protein activity. *Parasitology* 1996;112:221–5.
- [19] Cross H.F., Bronsvort B.M., Wahl G., Renz A., Achu-Kwi D., Trees A.J.. The entry of ivermectin and suramin into *Onchocerca ochengi* nodules. *Ann Trop Med Parasitol* 1997;91:393–401.
- [20] Strimmer K., von Haeseler A.. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. *Proc Natl Acad Sci USA* 1997;94:6815–9.
- [21] Felsenstein J.. Phylogeny Interference Package (Version 3.2). *Cladistics* 1989;5:164–6.
- [22] Bradley J.E., Helm R., Lahaise M., Maizels R.M.. cDNA clones of *Onchocerca volvulus* low molecular weight antigens provide immunologically specific diagnostic probes. *Mol Biochem Parasitol* 1991;46:219–27.
- [23] Schagger H., von Jagow G.. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987;166:368–79.
- [24] Hagen H.E., Klager S.L., Ham P.J.. A simple field method for the purification of *Onchocerca ochengi* microfilariae from a mixed *Onchocerca* infection in cattle. *Trop Med Parasitol* 1995;46:201–2.
- [25] Maizels R., Blaxter M., Robertson D., Selkirk S.. Parasite antigens, parasite genes. A laboratory manual for molecular parasitology. Cambridge: Cambridge University Press, 1991.
- [26] Kennedy M.W.. Structurally novel lipid-binding proteins. In: Kennedy M.W., Harnett W., editors. Parasitic nematodes. molecular biology, biochemistry, and immunology. CABI Publishing, 2001:309–30.
- [27] Gudas L., Sporn M.B., Roberts A.. Cellular biology and biochemistry of retinoids. In: Sporn M.M., Roberts A., Goodman D.S., editors. The retinoids. New York: Raven Press, 1994:443–520.
- [28] Wolff K.M., Scott A.L.. *Brugia Malayi*-retinoic acid uptake and localization. *Exp Parasitol* 1995;80:282–90.
- [29] Zahner H., Sani B.P., Shealy Y.F., Nitschmann A.. Antifilarial activities of synthetic and natural retinoids in vitro. *Trop Med Parasitol* 1989;40:322–6.
- [30] Lok J.B., Morris R.A., Sani B.P., Shealy Y.F., Donnelly J.J.. Synthetic and naturally occurring retinoids inhibit third- to fourth-stage larval development by *Onchocerca lienalis* in vitro. *Trop Med Parasitol* 1990;41:169–73.