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Expression and immune recognition of *Brugia malayi* VAL-1, a homologue of vespid venom allergens and *Ancylostoma* secreted proteins☆

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Abstract

Several important nematode parasites have been found to express members of a gene family variously termed as venom allergen antigen homologue (*vah*) or *Ancylostoma* secreted protein (*asp*). In some cases these products are secreted by infective larval stages and have been suggested to be effective vaccine immunogens. We isolated the corresponding gene from the human filarial nematode, *Brugia malayi*, by first searching the expressed sequence tag (EST) dataset generated by the Filarial Genome Project and then using gene-specific nondegenerate primers matching the selected gene for PCR, from *B. malayi* cDNA libraries. We report here the full-length gene sequence, which we have designated as *Bm-val*-1, for *vah/asp-like*. The corresponding protein (*Bm*-VAL-1) contains 232 amino acids in a single homology unit, unlike products from some other species in which there is a tandem repeat. A putative signal sequence is present at the 5′ end and there are two potential *N*-glycosylation sites. Murine antibodies to recombinant *Bm*-VAL-1 react with a 28 kDa protein in L3 extracts and recombinant *Bm*-VAL-1 is recognised by murine T cells primed with soluble L3 proteins. Of 82 ESTs corresponding to *Bm-val*-1, 72 are recorded from the infective larval (L3) stage. However, PCR on the first-strand cDNA from later mammalian stages revealed some expression at most subsequent time points. Over 95% (20/21) of microfilaraemic human filariasis patients are seropositive for antibodies to *Bm*-VAL-1, with particularly high levels of IgG3 and IgG4 isotypes. The IgG4 subclass may indicate stimulation by adult and/or microfilarial-derived immunogens. The association of *Bm*-VAL-1 with the infective stage and its recognition by humans exposed to filariasis suggests that further evaluation of this antigen as a vaccine candidate should be performed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Filariasis; Nematodes; Infective larvae; Isotypes; Vaccines

1. Introduction

The infective third-stage larvae of many parasitic nematodes undergo the most critical transition of the life cycle, from a free-living or arthropod-borne existence to the definitive, often mammalian, host [1,2]. Products from these stages are of particular interest as key mediators of the invasion process and as potential immunogens for anti-parasite vaccines [3–5]. When similar proteins are associated with larval stages of many different nematode species, a common pathway or essential component of the infection process may be inferred. One such instance is the group of proteins first described as *Ancylostoma caninum* secreted protein or ASP [6] with similarity to a wider family including, for example, hymenopteran venom allergens. Significantly, these products are released when resting larvae are stimulated to commence invasion. The venom allergen antigen homologue VAH/ASP family contains two dis-

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tinct forms, typified in *A. caninum* [7]: *Ac*-ASP-1 is a long-form 42 kDa protein while *Ac*-ASP-2 is a short-form 20–22 kDa product. Sequence analysis reveals that *Ac*-ASP-1 contains a tandem nonidentical repeat of an ancestral unit represented only once in *Ac*-ASP-2 [7].

Members of this gene family have now been reported from a range of nematode species. In the sheep intestinal nematode *Haemonchus contortus*, both the large-form Hc40 [8] and short-form Hc24 [9,10] have been identified. Multiple homologues have been discovered in the two human hookworm species *Ancylostoma duodenale* and *Necator americanus* [11,12], and also in *Meloidogyne incognita* [13], *Onchocerca volvulus* [14] and *Toxocara canis* [15]. Additional gene members in *Ascaris* and *Strongyloides* have also been indicated by hybridisation experiments with *Ac-asp*-2 probes [7]. Further, a homologue from the filarial nematode *Dirofilaria immitis* was selected by reactivity with immune dog serum (Tripp C, Wisnewski N, unpublished deposition) and a related gene in *Brugia malayi* reported from expressed sequence tag (EST) analysis [4]. Significantly, up to 17 homologous genes have been identified in the genome of *Caenorhabditis elegans* [7,12]. All but one of these are single-unit forms (e.g. *C. elegans* C39E9.1), the exception being F11C7.3 which displays a paired but nonidentical repeat structure [8].

More distant relatives of the VAH/ASP family can be found across a wide evolutionary spectrum. Within *A. caninum*, a related gene encodes a neutrophil inhibitory factor (NIF), which displays an antagonistic binding to the integrin receptor $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) on mammalian cells [16]. While the aforementioned insect venom allergens have no known function, they are similar to helothermine, a lizard salivary toxin, which blocks the ryanodine receptor [17]. In mammals, similar proteins have been described as the testis-specific proteins TPX-1, sperm-coating glycoprotein (SCG) and cysteine-rich secretory protein (CRISP) [18], as well as P25TI, a novel trypsin inhibitory protein upregulated in some tumour lines [19]. The larger gene family also includes an interesting set of plant defence proteins, all characterised by a conserved signature of key residues [7]. From these similarities, it is difficult to deduce a general function for nematode homologues, but it is attractive to postulate inhibition or activation of host receptor-dependent processes. For example, the *Ov*-ASP-1 and *Ov*-ASP-2 proteins from *Onchocerca volvulus* have been shown to promote corneal angiogenesis when injected into mice [14], a role in keeping with the formation of vascularised nodules in onchocerciasis.

Because vaccines against nematode parasites focus on the infective larval stage, heightened expression of *vah*/*asp* genes in larvae make these products an attractive candidate vaccine antigen [20]. Promising results have so far been obtained in two different species. Thus, four out of five sheep vaccinated with a fraction enriched with *H. contortus* Hc-24 were protected against establishment of adult parasites [21], while ASP-1-immunised mice show up to 80% reduction in infection [22,23].

The Filarial Genome Project has provided a major resource and impetus for gene discovery from *B. malayi* and other filarial nematodes [4,24–28]. EST sequences deposited for *B. malayi* include a number corresponding to a homologue of ASP [4,25,28]. We describe here the characterisation of this homologue, and its expression as a recombinant probe for immunological reactivity of patients infected with this major human tropical pathogen.

2. Materials and methods

2.1. Database searching

The ungapped TBLASTN algorithm was used with the *A. caninum* ASP-1 protein sequence matched against all deposits in the NCBI dbEST nucleotide database translated into all six frames [29]. Multiple ESTs with significant similarity were found, permitting a putative full-length sequence to be assembled. From this, a 5′ gene-specific primer was designed to amplify the gene by PCR from an L3 cDNA library, 5′-*AGT ACT TAC TAG A*CG ACA TCT TAC TGT T-3′ (roman typeface nt −68 to −54, 5′ of the start codon), in conjunction with M13 reverse primer for a 3′ vector sequence. This product was cloned and sequenced in full.

2.2. PCR amplifications and 5′ RACE

The 5′ end of the *Bm-val*-1 cDNA was amplified using the rapid amplification of cDNA ends (RACE) procedure following the supplier's instructions (Gibco-BRL). Starting with mixed adult *B. malayi* total RNA, first-strand cDNA was synthesised from a primer sequence close to the 5′ end of the cDNA clone; this primer was 5′-TAG TCG ACC TCC TGG ACA TTC-3′ (complementary to nt 72–52 of cDNA in which the initiating ATG codon is numbered 1–3). PCR was then performed with an anchor primer supplied in the kit and a second gene specific primer: 5′-AAC GAA AAA TAA CAT TTT GCC GAG-3′ (complementary to nt 15 to −9). The PCR products were ligated into the pMOS-blue vector. Clones were picked at random and fully sequenced.

2.3. Reverse transcription-PCR

First-strand cDNA was produced from total RNA using GeneAmp RT-PCR kits (Applied Biosystems, Cheshire, UK) as previously described [5]. For PCR,

val-1- specific primers were used on first-strand cDNA for 30 cycles of 1 min at 99 °C, 1 min at 55 °C and 1.5 min at 72 °C. The primers used were *A*TT TGA ATG TCC AGG AGG TCG A, corresponding to nt 49–69, and *T*TT TTC TAC ACA ATC CAG ATT T, corresponding to nt 660–640; in each case an additional nucleotide (italicised) was added at the 5′ end.

2.4. Expression and antibody analysis

Recombinant *Bm*-VAL-1 protein was expressed in *E. coli* by cloning the presumed mature coding sequence (a 612 bp insert encoding aa 17–220) into the pET-29T vector (Novagen), following PCR with the two primers described above (49–69 and 660–640). Protein expression was induced in BL21(DE3) cells with 1 mM IPTG for 3 h at 37 °C. Bacteria were pelleted and sonicated in a 6 M urea buffer suitable for metal chelating chromatography on His-bind resin (Novagen). Eluted protein was dialysed against PBS, and recovered at a final concentration of 2 mg ml^{-1}. Both BALB/c and CBA/Ca mouse strains were immunised with two doses of 10 μg r*Bm*-VAL-1 (first in complete Freund's adjuvant, the second in incomplete), one month apart. Serum samples taken 7 and 14 days following the boost showed that only the BALB/c responded with a polyclonal antibody response. For Western blots, whole microfilarial (Mf), L3 and adult worms were solublised directly in PBS, or 1.5% *n*-octylglucoside (*n*OG, Sigma), or in SDS-PAGE loading buffer. Samples were subjected to SDS-PAGE and electrotransfer, then probed with a 1/200 dilution of murine anti-*Bm*-VAL-1 or normal mouse serum, followed by peroxidase anti-mouse Ig (BioRad, 1/2000) and ECL substrate.

2.5. Murine T cell reactivity

BALB/c mice were immunised with 10 μg of soluble extract of L3 mixed with an equal volume of complete Freund's adjuvant (Sigma) in the rear footpad. Ten days later, popliteal lymph node cells were recovered and cultured as described in detail elsewhere [30]. The data were compared by the two-tailed *t*-test and a *P* value of 0.05 or less was considered significant.

2.6. ELISA

Sera from 42 adult individuals residing in the Palu Valley area of Central Sulawesi, Indonesia, which is endemic for *B. malayi* filariasis, were tested for isotype specific antibodies to recombinant *Bm*-VAL-1. Of these patients, 21 were patent microfilaraemic (range 40–3300 Mf ml^{-1}) and 21 asymptomatic amicrofilaraemic (exposed but 'endemic normal') individuals. Recombinant antigen was coated onto Nunc immunoplates at 1 μg ml^{-1} in 0.06 M carbonate buffer and plates were otherwise treated as described previously for analyses of responses to whole *B. malayi* antigens [31,32].

3. Results and discussion

3.1. Search of dbEST for ASP homologues

The *A. caninum* ASP-1 sequence was used to screen the NCBI dbEST database containing the Filarial Genome Project EST sequences from multiple stages of *B. malayi*. Of approximately 18 700 sequences, 82 showed high levels of similarity and 72 of these were from the infective L3 stage larval cDNA library. This represents an abundance of 2.22% (72/3249 L3 ESTs). Similar levels of abundance have been observed for the related nematode *O. volvulus* [14]. Three more cDNAs were noted from the Mf stage, five from post-infection larvae and two from adult male worms [28]. Closer inspection of these sequences indicated that all were derived from the same gene, which has been assigned a single cluster number (BMC00351) by the Filarial Genome Project.

3.2. PCR amplification and 5′ RACE

The longest EST contained an open reading frame of 220 amino acids, equivalent to a single homology unit of *Ac*-ASP-1. As *Ac*-ASP-1 and *H. contortus* Hc40 were known to contain tandemly duplicated domains, the possibility that *Bm-val*-1 encodes a larger protein was investigated. From first-strand cDNA of mixed adult-stage parasites, a primer close to the known 5′ end was used for 5′ RACE. However, no longer product was obtained in the two experiments, although the same protocol and reagents were successful for other transcripts [33]. No product was obtained in amplifications with the nematode 22-nt spliced leader and a *Bm-val*-1 specific primer, although parallel conditions amplified a number of other larval cDNAs [2]. All ESTs for which appropriate sequence had been recorded encoded a stop codon at nt 661–663 indicating that no second 3′ domain exists.

3.3. Sequence of B. malayi vespid (venom allergen)/Ancylostoma (secreted protein)-like product (Bm-VAL-1)

The open reading frame encodes 220 amino acids, of which the first 16 represent a putative signal sequence. The presence of this hydrophobic motif further indicates that the sequence represented here is the authentic full-length product. This sequence differs at two nucleotide positions from that deposited by McCarthy and Hopkins (AF042088). These substitutions are each synonymous and occur at nt 219 and 351. Of 17 ESTs for

which data are available, 11 (65%) have the two nucleotides reported by McCarthy and six (35%) have the two we describe here. Such differences may well represent allelic forms among the *B. malayi* gene pool, as noted at other loci [34]. This indication of sequence polymorphisms is not surprising in the light of Qiang et al.'s recent report of no less than 42 nucleotide variations (of which 12 were nonsynonymous) among *Ac-asp*-1 genes from different *A. caninum* isolates [35]. Other features of the protein sequence include two *N*-glycosylation sites and a preponderance of cysteine residues in the C-terminal segment (aa 164–220), which contains seven of the ten cysteines in the protein.

3.4. Evolutionary relationship of VAH/ASP-like (VAL) family members

The nematode VAL proteins show characteristics of a larger protein family, designated pfam00188 and also known as SCP (sperm coating protein)-like with homologues of mammalian, insect, fungal and plant origins. The same family has also been named CAP, to denote Cysteine-rich secretory proteins of mammals, vespid venom Allergens, and plant Pathogenesis-related proteins [36]. *Bm*-VAL-1 shares 17 of the 19 residues identified as conserved across the whole CAP family (Fig. 1).

Cladograms summarising the likely phylogeny of these genes have recently been published in which all the filarial VAL sequences form a monophyletic group distinct from other nematode genes, with 97–100% bootstrap support [12,14]. Within the filarial nematodes, *Bm*-VAL-1 is most similar to *Wb*-VAH-1, sharing 91% amino acid identity (Fig. 1). Other filarial VAL sequences in the public databases include one from the dog heartworm (*D. immitis*) and three homologues from *O. volvulus*, of which only *Ov*-ASP-3 is expressed by the L3 stage [14]. ESTs for a VAL homologue are also available for *Litomosoides sigmodontis* [37]. The complete filarial sequences, together with the canonical *A. caninum* ASP and a representative venom allergen from the hymenopteran fire ant, are also compared in Fig. 1.

3.5. Expression by different life cycle stages

The pattern of expression of *Bm-val*-1 can be followed through the mammalian life cycle of *B. malayi* by RT-PCR from first-strand cDNAs taken at 1- to 2-day intervals over the first 4 weeks' infection of jirds from L3 to the adult stage. The mRNA is strongly represented in the mosquito-borne L3 stage, as well as at certain other life cycle points such as maturing L4 and young adult worms (Fig. 2). Moreover, expression occurs in the Mf stage as reflected in ESTs derived from

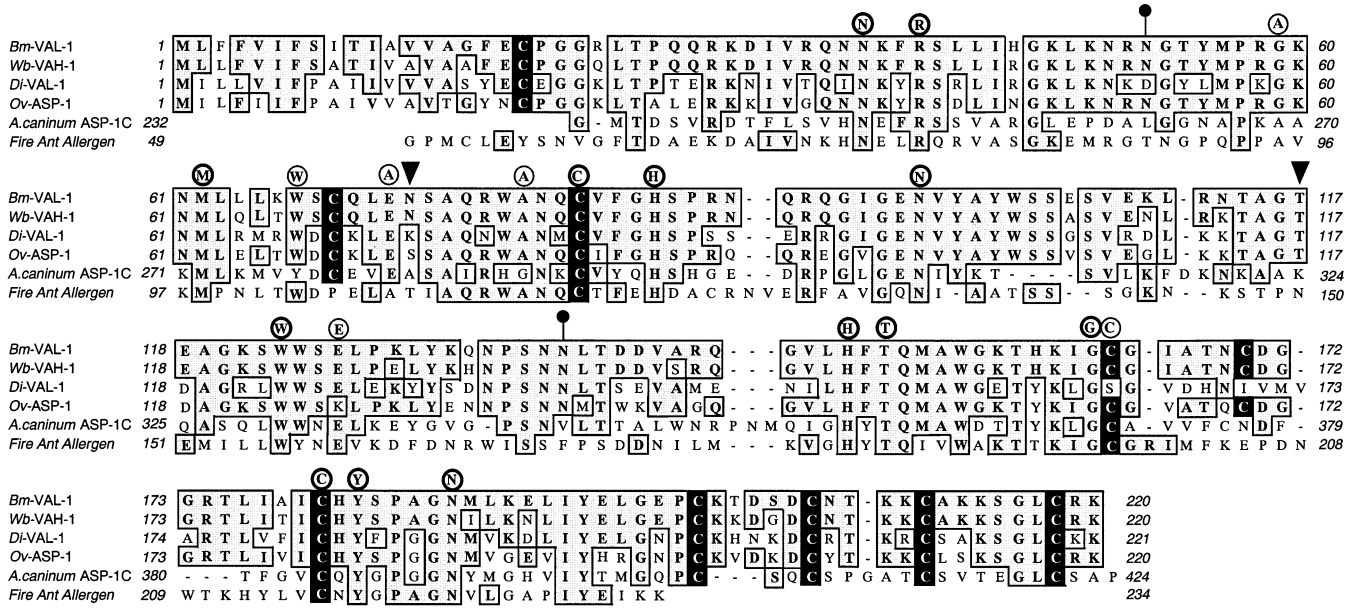

Fig. 1. Alignment of filarial VAL proteins with homologues from *Ancylostoma* and the fire ant. Authors' designations of genes as VAH or ASP have been respected. Alignments were prepared using the ClustalW facility on MacVector 7.0. All cysteine residues are displayed in black. Lollipop symbols identify the two potential *N*-glycosylation sites, both of which are shared with *Wb*-VAH-1, and one of which is present in *Di*-VAL-1. Inverted triangles denote codons found to show synonymous nucleotide polymorphisms (nt 217/219, AAC/AAT, both Asn; and nt 349/351 ACC/ACG, both Thr). Residues identified as characteristic of the CAP family from mammals, insects, nematodes and plants [35] are circled above the alignment, heavier shading denoting conservation within the nematode sequences shown. Accession numbers of the sequences presented are as follows: *W. bancrofti* VAH-1, AF109794; *Dirofilaria immitis* VAL, AF001100; *O. volvulus* ASP-1, AF020586; *A. caninum* ASP-1, AF132291; *Solonepsis invicta* (fire ant) allergen sol i 3, AF012919.

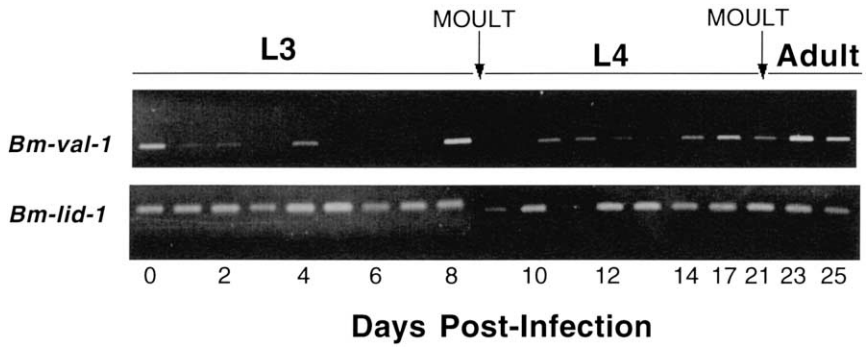

Fig. 2. Expression of *Bm-val*-1 during the mammalian stages of the *B. malayi* life cycle. The stage and timing of the moults following infection of the mammalian host are indicated above the panel showing PCR products amplified from each time point. d0 represents mosquito-derived L3. mRNA taken at each time point was used to make first-strand cDNA as templates in PCR reactions using the *Bm-val*-1-specific primer pair detailed in Section 2. As a control, primers for *Bm-lid*-1 (a homologue of Ov-L3 from *O. volvulus*, Murray J, Maizels RM, unpublished) show constant expression from all stages tested.

the Mf library. Thus, transcription does not show complete developmental restriction.

3.6. Native Bm-VAL-1 is a 28 kDa L3 protein

Expression of the *Bm*-VAL-1 protein was also monitored using a polyclonal antibody raised to the recombinant protein. Western blot analysis with PBS-soluble extracts of the three major life cycle stages (Mf, L3 and adults) reveals antibody-reactive proteins in both Mf and L3 (Fig. 3A). The L3 component has an estimated molecular weight (mol. wt.) of 28 kDa. A strong Mf band can also be seen with a slightly faster mobility, but no corresponding antigen is present in adult extracts. Interestingly, some fainter bands are apparent in the higher mol. wt. ranges. Since many nematode species are known to encode multiple VAL-type genes (e.g. two in *Haemonchus*, ≥ 9 in *Necator*, 17 in *C. elegans*), it is possible that these weaker reactions represent related but distinct VAL gene products.

While *Bm*-VAL-1 protein is present in PBS-soluble extracts, some of the product requires detergent for its release (Fig. 3B). Moreover, the harsher conditions of SDS-2ME solubilise a reactive form of approximately 40 kDa. Again, comparison with other species suggests that this may represent a different member of the same gene family in *B. malayi*.

Several experiments were undertaken to establish if *Bm*-VAL-1 is secreted by infective larvae. Overnight incubation of mosquito-derived L3 in a variety of conditions (1000 L3 ml^{-1} of RPMI or Grace's medium at 26 or 37 °C) did not result in the release of sufficient *Bm*-VAL-1 protein to be detected by Western blot, although another highly-expressed larval protein (*Bm*-ALT-1) could readily be observed under these conditions. If *Brugia* larvae, like *Ancylostoma* larvae, require a developmental cue to stimulate release of the ASP-like VAL protein, it could be argued that only exposure to mammalian factors in vivo can provide the necessary signal. Thus, the question of whether *Bm*-VAL-1 functions as an exo-antigen within the host environment remains to be resolved.

3.7. Expression and reactivity of Bm-VAL-1

A panel of human filariasis sera was tested for antibody recognition of recombinant *Bm*-VAL-1-protein and high levels of serological reactivity were observed (Fig. 4). Two groups of individuals were studied: asymptomatic amicrofilaraemic persons, who are clinically normal but exposed and may harbour cryptic infections (Mf −); and microfilaraemic patients with circulating parasites (Mf +). In the first subset of endemic normals, 18/21 (86%) were seropositive in at least one of the 4 IgG subclasses of antibodies to *Bm*-VAL-1, while in Mf + patients, the proportion was 20/21 (95%). The most vigorous antibody isotypes observed were IgG3 and IgG4.

The IgG3 isotype has previously been linked to protective immunity to the L3 stage [38–40], while the presence of IgG4 is strongly associated with exposure to adult worms and microfilariae [41]. Because previous work suggests that larval antigens do not elicit IgG4 responses [32], the presence of anti-*Bm*-VAL-1 IgG4 may reflect a low level of expression by adult and microfilarial stages.

3.8. Immunogenicity to T cell populations

To test whether T cell responses were likely to be mounted to VAL, mice were immunised with soluble proteins from the infective larval stage. When primed lymph node cells were challenged in vitro with r*Bm*-VAL-1, a significant proliferative response was observed in every experiment (Fig. 5; $P < 0.005$). Assays for antigen-specific cytokine release showed that levels of IL-4 and IFN-γ produced in response to *Bm*-VAL-1 were only marginally above background (no-antigen

control) values (data not shown). Human T cell responses were also gauged by stimulation of PBMC from filariasis patients. Positive proliferative responses were observed in some patients, but in only one case was significant production of IFN-γ noted (Sartono E, Yazdanbakhsh M, personal communication).

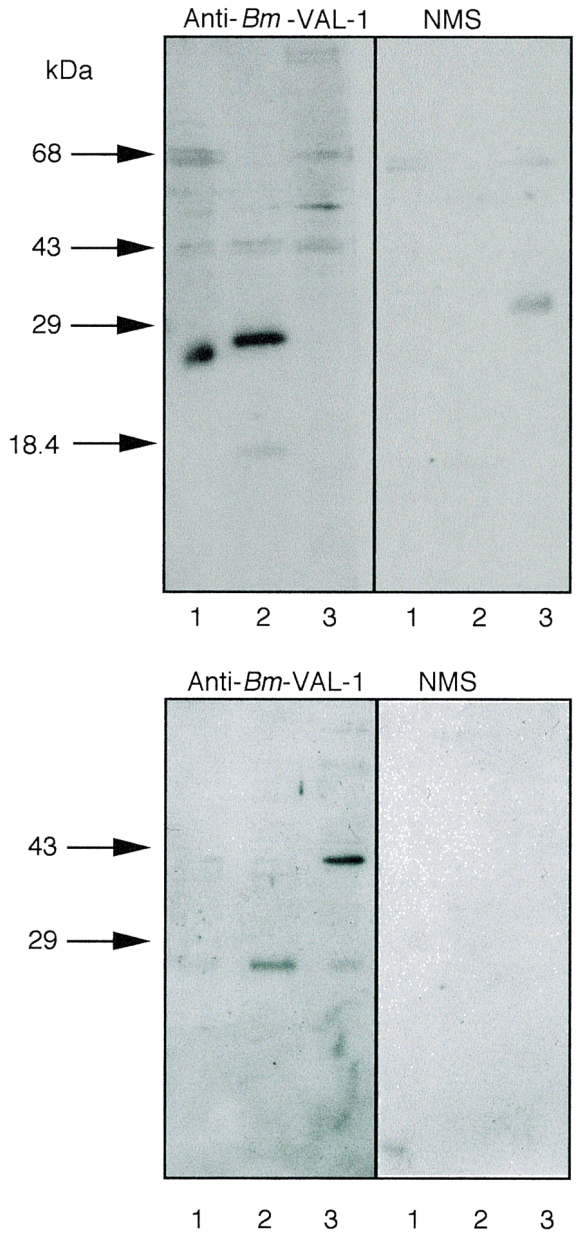

Fig. 3. Western Blot identification of native *Bm*-VAL-1 antigens. (A) Mf (track 1) and L3 (track 2) 10 μg of PBS-soluble extracts were probed with polyclonal mouse antibody to recombinant *Bm*-VAL-1 (left panel). No reaction was observed with adult extracts (track 3) or with normal mouse serum (NMS, right panel). (B) L3 extracted sequentially with PBS (track 1), *n*OG (track 2) and SDS-PAGE loading buffer (track 3) were probed with mouse antibody to r*Bm*-VAL-1 (left panel). No reaction was observed with NMS (right panel).

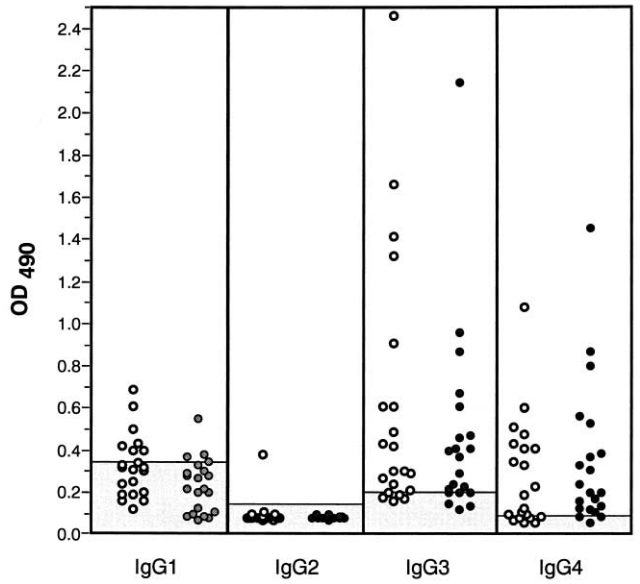

Fig. 4. Specific antibody responses in human filariasis patients. Antibody levels specific for *Bm*-VAL-1 in the sera of 21 Mf-negative endemic normal subjects (○) and 21 microfilaraemic patients (●) were measured by ELISA for IgG1, IgG2, IgG3 and IgG4 using plates coated with 1 μg ml^{-1} recombinant antigen. All patients were adults residing in an area of Central Sulawesi, Indonesia, endemic for *B. malayi*. The cut-off values are represented by the shaded boxes and are derived from the mean of three European normal control values plus three SD.

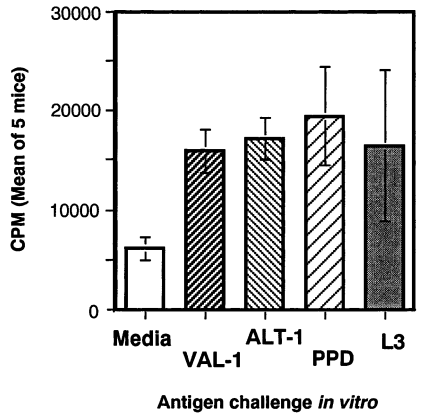

Fig. 5. T cells from L3-immunised mice respond to *Bm*-VAL-1. BALB/c mice were immunised with 10 μg of soluble L3 extract proteins in complete Freund's adjuvant subcutaneously in the footpad. Ten days later, popliteal lymph node cells were taken and cultured in vitro in the presence of 10 μg ml^{-1} recombinant VAL protein. Positive controls included *Bm*-ALT-1 (an abundant L3 protein [5]), mycobacterial PPD (a constituent of complete Freund's adjuvant) and the L3 extract used for initial immunisation. Data represent the means and standard deviations from groups of five mice. The response to *Bm*-VAL-1 was significantly above media-alone controls by a two-tailed *t*-test ($P < 0.005$).

3.9. Vaccination

Because of the prominence of *Bm-val*-1 among larval cDNA transcripts and the success of similar products in vaccination against other nematode species, recombinant *Bm*-VAL-1 was used to immunise jirds. Following four injections (the first of 75 μg in complete Freund's adjuvant, two boosts of 25 μg in incomplete Freund's adjuvant and a final boost of 7.5 μg in IFA), jirds were challenged with 300 mosquito-derived infective larvae intraperitoneally. Four weeks later, the *Bm*-VAL-1-immunised group showed a mean reduction of 64% in parasite recovery from the peritoneal cavity (immunised: geometric mean 18.6, range 7–108; control given adjuvant alone: geometric mean 50.9, range 13–195). Although a substantial difference, this failed to reach statistical significance (Mann–Whitney nonparametric test, group sizes = 6). A similar experiment was then conducted, but again, no significant effect was observed and only minimal reductions in recovery obtained. Future work may aim to refine the immunisation protocol and to combine *Bm*-VAL-1 with other candidate immunogens in a multivalent vaccine.

Vaccination against filarial nematodes offers a robust strategy for eradication of this major tropical scourge [42–44]. Recently, the recombinant *B. malayi* larval-specific antigen *Bm*-ALT-1 was reported to elicit protective responses in jirds [5]. The expression of *Bm*-VAL-1 is less stringently restricted to the L3 than is the case for *Bm*-ALT-1 and the possibility remains that VAL immunisation could prime pathogenic responses against mature stages. Future vaccination studies will need also to assess whether vigorous immune responses to nematode VAL proteins carry any risk of harmful reactivities either to environmental antigens (such as may be encountered from insect bites), or to the endogenous proteins in mammalian hosts such as the testis-specific autoantigen Tpx [45].

4. Conclusions

The VAL family has become one of the most intensively studied sets of genes from nematode parasites, showing strong association with larval invasion of the mammalian host. We report here that these products do not go unnoticed by the host immune system and suggest that an appropriate response to these antigens may prove to be protective. It will now be important to establish the biological function of the VAL proteins in the host–parasite interaction, so that we can begin to appreciate a fuller picture of how VAL products may promote parasite infection and how immune responses to these antigens may frustrate parasite survival.

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