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Citation for published version:
<http://iai.asm.org/content/67/12/6329.abstract>

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

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Comparative Analysis of Glycosylated and Nonglycosylated Filarial Homologues of the 20-Kilodalton Retinol Binding Protein from Onchocerca volvulus (Ov20)

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Received 30 June 1999/Returned for modification 16 August 1999/Accepted 9 September 1999

Ov20 is a structurally novel 20-kDa retinol binding protein secreted by Onchocerca volvulus. Immunological and biological investigation of this protein has been hampered by the inability to maintain O. volvulus in a laboratory setting. In an effort to find a system more amenable to laboratory investigation, we have cloned, sequenced, and expressed cDNA encoding homologues of Ov20 from two closely related filarial species, Brugia malayi (Bm20) and Acanthocheilonema viteae (Av20). Sequence comparisons have highlighted differences in glycosylation of the homologues. We present here an analysis of mouse immune responses to Ov20, Bm20, and Av20. The results suggest a strong genetic restriction in response to native Bm20 that is overcome when recombinant, nonnative material is used. Reactivity of human filarial sera to the three recombinant proteins confirmed previous specificity studies with Ov20 but highlighted important differences in the reactivity patterns of the O. volvulus and B. malayi homologues that may be due to differences in glycosylation patterns. Ov20 is a dominant antigen in infected individuals, while Bm20 is not. The availability of the B. malayi homologue enabled us to use defined murine reagents and inbred strains for genetic analysis of responsiveness in a way that is not possible for Ov20. However, the close sequence similarity between Ov20 and Av20 suggests that the A. viteae model may be more suited to the investigation of the biological functions of Ov20.

Infection of humans by parasitic filariae leads to a spectrum of clinical manifestations ranging from a remarkable absence of responsiveness to severe immunopathological conditions like elephantiasis and irreversible blindness (17, 21, 22). The final outcome of an infection, whether it be protective immunity, tolerance, or disease development, is largely dependent on the way in which the host immune system recognizes and responds to these multicellular organisms (16, 21, 22). To understand and unravel these complex immunological interactions between parasites and their mammalian hosts, it is crucial to look at specific responses to individual antigens.

Ov20 is an immunodominant glycoprotein antigen of Onchocerca volvulus, the causative agent of river blindness in humans. A major part of the clone (OvMBF20/11) has shown a high degree of specificity to onchocerciasis sera, with sera from related nematodes failing to react despite homologues of this protein being present in the other species (3, 4). The protein has been incorporated in a serodiagnostic tool to analyze human immune responses to Onchocerca infection (4, 5, 24). Fluorescence-based ligand binding assays show the protein to contain a high-affinity ligand binding site for retinol; algorithms which predict secondary structure indicate a predominance of alpha helices, with no evidence of the beta structures seen in retinol binding proteins characterized so far (15). Ov20, thus represents a new class of helix-rich retinol binding protein of unknown function and appears to be confined to nematodes (15, 31).

The full-length cDNA corresponding to Ov20 has been isolated, and extensive database searches failed to detect similarity to proteins of known function; however, its antigenic and sequence conservation in a wide range of nematodes suggests an important biological role (31). The only other family of retinol binding proteins of similar size and helicity are individual units of the polypeptide allergens of nematodes such as ABA-1 of Ascaris suum and gp15/400 of Brugia malayi (13, 14).

Retinoids have been implicated in a variety of biological functions in vertebrate and nonvertebrate systems (6, 7, 11, 12, 29). O. volvulus is known to sequester retinol to a concentration eight times in excess of the surrounding host tissue (30). Initial clinical manifestations of onchocercal eye damage include night blindness, a symptom consistent with retinol deficiency (25). Although the in vivo function of Ov20 with respect to both the worm and the host is yet to be established, retinol segregation, initial symptoms compatible with retinol deficiency, and secretion of a unique immunodominant retinol binding protein suggest that this molecule is worthy of further investigation. The lack of an animal model for onchocerciasis has hampered further characterization of Ov20, creating a need for homologues of this molecule to be cloned and expressed in parasite systems where animal work is possible. We selected the human filarial parasite B. malayi, the causative agent of lymphatic filariasis, and the rodent filarial parasite Acanthocheilonema viteae, which because of its close phylogenetic relationship to O. volvulus has been considered a model for onchocerciasis.

The objectives of this study were twofold: first, to identify by cloning of the homologous proteins a reliable candidate for modelling the immune response to Ov20 and second, to analyze the immune reactivity of infected mice and humans to the different recombinants to elucidate differences in protein structure that may influence immune responsiveness. Here we report on the isolation and expression of cDNA encoding the homologues of Ov20 in B. malayi (Bm20) and A. viteae (Av20).
Sequence comparisons highlighted differences in the pattern of glycosylation in the homologues, allowing evaluation of the role of glycosylation in antigen recognition. Further, the availability of sera from inbred mice inoculated with *B. malayi* allowed a genetic analysis of responsiveness that would not be possible for Ov20.

**MATERIALS AND METHODS**

**Cloning of Av20.** DNA encoding the *A. vitae* homologue was amplified from an adult cDNA library with PCR and cloned into pET-15b (Novagen, Madison, Wis.), with an intermediate cloning into pUC18. The oligonucleotide primers for Av20 used for the PCR (Ov20HTF [5′ CTC CAT AGT GCA AAT GTT GTT CCG TTT TC3] and Ov20HTR [5′ CGGATCTCGATTTTGGCTG ACC 3′]) were designed to generate a protein starting from the predicted cleavage site for the signal peptide, Asn17, of the Ov20 sequence. The inserts which were originally cloned into Smal-digested pUC18 by blunt-end ligation and transformed into the Escherichia coli DH5α were subcloned into pET-15b by using the NdeI and BamHI sites.

**Cloning of Bm20.** Oligonucleotides derived from the Ov20 sequence were used to find the *Brugia* homologues. To obtain the full-length *Brugia* sequence, the upstream primer was used with an oligo (dT) primer, and the downstream primer was used with a primer for the spliced leader sequence (for *B. malayi* transcripts). Template DNA was derived from *Brugia* adult or microfilarial cDNA amplified with the splashed leader and oligo (dT) primers courtesy of Bill Gregory. Sequences were also obtained from commercial microfilarial and adventitious DNA libraries provided by Steve Williams, Smith College. Oligonucleotides included XbaI, NdeI, Pfu, and BamHI sites for ease of cloning. *Oligonucleotides used were Ov20XN (TTTCTAGAACCATGGCAAATGTTGTTCCTG), Ov20PB (CCGGATCTCGATTTTGGCTGACC 3′), and a DNA sequence, BM20XN (TTTCTAGAACCATGGCAAATGTTGTTCCTG), which was originally cloned into pUC18. The oligonucleotide primers for the PCR (Ov20HTF [5′ CTC CAT ATG GCA AAT GTT GTT] and Ov20HTR [5′ CGGATCTCGATTTTGGCTGACC 3′]) were designed to generate a protein starting from the predicted cleavage site for the signal peptide, Asn17, of the Ov20 sequence. The inserts which were originally cloned into Smal-digested pUC18 by blunt-end ligation and transformed into the Escherichia coli DH5α were subcloned into pET-15b by using the NdeI and BamHI sites.

**Expression and purification of Bm20, Av20, and Ov20.** Bm20 and Av20 were expressed in the *Escherichia coli* strain DH5α, which allows expression of the polyhistidine domain to facilitate purification. Expression and purification involved the use of step gradients. Expression of Av20 in the pET-15b expression vector utilized the polyhistidine domain to facilitate purification. Sequence comparisons highlighted differences in the pattern of glycosylation. The fragment representing Ov11 is highlighted.

**RESULTS**

**Sequence comparison.** Homologues of Ov20 were cloned from *B. malayi* and *A. vitae* and sequenced for comparison with the *Onchocerca* molecule. Expression and purification of the recombinant homologues allowed the immunological analysis detailed below. Sequence comparison of Ov20, Bm20, and Av20 showed Ov20 and Av20 to be very similar, sharing three N-linked glycosylation sites (NXS/T) and differing in only nine amino acids (97% similarity) (Fig. 1). The codons for the three glycosylation sites were identical except for the presence of a serine residue at position 46 in Av20 instead of a threonine as in Ov20. The Bm20 sequence was more divergent, with 26 amino acid differences from Ov20 (91% similarity), resulting in the loss of all three glycosylation sites found in Ov20. However, a new N-linked glycosylation site was located near the carboxy terminus. It is unlikely that this site is used, as previous studies

**DNA cloning.** A new N-linked glycosylation site was located near the carboxy terminus, and areas of homology among the three recombinants are indicated (**). The fragment representing Ov11 is highlighted.

**FIG. 1. Alignment of the predicted amino acid sequences of the retinol binding proteins Bm20, Ov20, and Av20.** N-linked glycosylation sites are underlined, and areas of homology among the three recombinants are indicated (**). The fragment representing Ov11 is highlighted.

**DNA cloning.** A new N-linked glycosylation site was located near the carboxy terminus, and areas of homology among the three recombinants are indicated (**). The fragment representing Ov11 is highlighted.
have shown no evidence that Bm20 is glycosylated (1a, 31). The signal peptide sequence in Ov20 homologue. The mature Bm20 and Av20 proteins were predicted to have molecular masses of 18.545 and 18.648 kDa, respectively.

**Serological responses in mice exposed to live parasites or recombinant proteins.** We have previously done extensive analysis of immune responses in mice implanted with *B. malayi* (1, 19, 20). This provided the necessary reagents for analysis of serum reactivity to Bm20. Sera from mice implanted intraperitoneally with live adult *B. malayi* were assayed for reactivity to *B. malayi* antigens Bm20 and whole *B. malayi* antigen, with maltose binding protein (MBP) serving as a nonspecific control. While the BALB/c (H-2^d^), CBA (H-2^k^), BALB.k (H-2^k^) mouse strains failed to show significant reactivity to Bm20, C57BL/6 (H-2^b^) mice showed a high reactivity that was significantly different from background MBP (P < 0.01) (Fig. 2). The results suggest a strong genetic restriction in response to native Bm20 as had previously been observed for gp15/400. The genetic restriction observed for gp15/400 was shown to have both H-2 and non-H-2 components (1). To assess the role of H-2 genes in the Bm20 responsiveness, BALB.b (H-2^b^) mice were implanted with adult *Brugia* parasites and found to produce antibody to Bm20, suggesting that the response is at least in part H-2 restricted (data not shown). When the Bm20-reactive C57BL/6 serum was tested against all three recombinant proteins, Av20 and Ov20 were not recognized despite their sequence similarity to Bm20 (Fig. 3). This finding confirms previous specificity studies with Ov20 (4).

Sera from nonresponder mice (B10.D2, BALB.k, BALB/c, and CBA) immunized with *B. malayi* parasite extract in Freund's complete adjuvant also failed to generate an antibody response to Bm20 (data not shown). In contrast, when the studies were repeated with sera from mice immunized with recombinant Bm20, all mouse strains tested (BALB/c, C57BL/6, and B10.D2) exhibited a strong recognition of Bm20 and of Ov20 and Av20 (Fig. 4). Thus, the genetic restriction and species specificity are overcome when recombinant, non-native material is used.

Sera from mice immunized with Ov20 had a similar pattern of recognition to the three recombinants; however, sera from mice immunized with Ov11, a truncated version of Ov20 (Fig. 1) that has shown a high degree of specificity for onchocerciasis sera (1), showed a different recognition pattern. The anti-Ov11 sera reacted significantly (P < 0.001) with Av20 and Ov20 in comparison to normal serum controls but responded poorly to Bm20 (Fig. 4).

**Serological responses of human filarial sera.** In *B. malayi*, a 15-kDa retinol and lipid binding polypeptide allergen (gp15/400) which is immunodominant in elephantiasis has been characterized (1, 14, 23). One of the objectives of this report was to establish whether Bm20 was an immunogen in human filarial infection. The reactivity of the three proteins with a panel of human onchocerciasis and lymphatic filariasis sera was tested.

The lymphatic filariasis sera included sera from both *B. malayi* - and *W. bancrofti*-infected individuals. *B. malayi* and *W. bancrofti* are very closely related both phylogenetically and pathologically. They both cause mosquito-borne diseases with identical worm locations in the mammalian host and cause very similar clinical symptoms. Ov20 and Av20 were recognized strongly by onchocerciasis sera (P < 0.001) but not by lymphatic filariasis sera, confirming previous studies on the specificity of Ov20 (Fig. 5) (4). In contrast to Ov20 recognition by onchocerciasis sera, Bm20 recognition by *B. malayi* sera was only marginally significant despite the presence of the homologue in the parasite. *W. bancrofti* sera showed no significant reactivity to any of the three proteins. The slight differences in reactivity between *B. malayi* and *W. bancrofti* is most likely due to differences in geographic localization, as no significant reactivity to Bm20 was observed in 28 *B. malayi* sera representing four clinical categories from Sumatra, Indonesia (data not shown). In all cases, the sera showed strong reactivity to whole parasite extract.

**DISCUSSION**

Sequence comparison of the Ov20 homologues showed close similarity between Ov20 and Av20 at both nucleotide and amino acid levels, while the Bm20 sequence was more diver-
Significantly, major differences were seen in the coding for N-linked glycosylation sites. Three shared sites in Ov20 and Av20 were replaced by a single new site in Bm20. The closer relationship between Ov20 and Av20 compared to Bm20 is not supported by phylogenetic analysis based on small-subunit rRNA sequencing of *O. volvulus*, *B. malayi*, and *A. vitaeae* (2a), suggesting that the changes may reflect important functional differences between these molecules in the different organisms.

The secondary structure predicted for Ov20 is a short alpha helix at the N terminus, corresponding to amino acids 30 to 46 of Ov20, followed by three longer alpha helices (15, 26). Two copies of a degenerate repeat (Ile-Pro-Glu-tyr/Val-Lys/Asn/Glu-Phe) seen in the region corresponding to the short alpha helix of Ov20 also appears to be conserved in the *B. malayi* and *A. vitaeae* homologues. The presence of a signal peptide in both Ov20 and Bm20 suggests that the proteins are secreted, but the localization of Bm20 has not been confirmed. Immunoprecipitation studies of the Av20 model have confirmed secretion in all life cycle stages (21a).

In this study, four inbred strains of mice were exposed to Bm20 in three contexts: by infection with live parasites, by adjuvant-assisted immunization with the native molecule, and by immunization with a recombinant molecule. Our results show that the antibody responses to the native molecule are controlled by factors within the H2 complex but that the restriction observed was overcome by immunization with the *E. coli*-derived recombinant protein. We have previously observed a high level of genetic restriction in the murine response to the polypeptide antigen gp15/400 of *B. malayi* at both antibody and T-cell levels (1). These results suggest that features of the native molecule interfere with its ability to be recognized by host T cells.

We had originally proposed that glycosylation alters processing or presentation of the antigen, but the absence of glycosylation in native Bm20 suggests that the restricted response is determined by other factors. It is intriguing to consider that Bm20 and gp15/400, both retinol binding proteins, may bind similar ligands in vivo, which may affect processing or recognition of these molecules. Sera from mice immunized with Ov20 and Bm20 *E. coli* recombinant molecules recognized all three homologues (Ov20, Av20, and Bm20). In contrast, human onchocerciasis sera recognized only Ov20 and Av20, con-
sistent with the close sequence relationship between these two proteins. The difference in reactivity to Bm20 observed between mice immunized with Ov20 and its specific fragment Ov20/11 suggests that the central portion of the molecule determines species specificity (Fig. 4). The close relationship between Ov20 and Av20 suggests that Av20 could be used in an A. viteae infection model in mice to investigate the biological functions of Ov20, which have up to now been hampered by the inability to maintain O. volvulus in a laboratory setting.

The reactivity patterns of Bm20, however, were distinct from those of the other molecules, as sera from patients infected with B. malayi failed to recognize it, suggesting that antigenic epitopes are not exposed to the immune system during the natural course of the infection in humans. However, the ability of C57BL/6 to respond strongly to Bm20 during live infection suggests that antigenic epitopes are in fact accessible and that the inability to maintain A. viteae proteins. The difference in reactivity to Bm20 observed be-
sistent with the close sequence relationship between these two proteins, as sera from patients infected with B. malayi is apparently not. It is unlikely that sequence differences alone could explain the non-
responsiveness of Brugia to Bm20.

The possible role of glycosylation in immunomodulation is an interesting area of future study. Carbohydrates may be important in determining which epitopes are processed or presented to the immune system (33), and the differential glyco-
sylation observed in the three molecules may contribute to the differential reactivity observed. Furthermore, glycosylation may determine extracellular stability, as one of the key func-
tions of glycosylation is to protect proteins from proteolytic degradation; i.e., Bm20 may be secreted but more rapidly de-
graded. Yet another possible explanation is the presence of a sheath in B. malayi microfilariae which could impede secretion of the protein into host tissue and thereby reduce tissue Bm20 levels significantly. It is intriguing to consider the possibility that gp15/400, a protein which has a profile of retinol binding similar to those of Ov20 (14) and Bm20 (12a) and is both immunodominant and glycosylated, may play the role of Ov20 in Brugia infection.

The function of Ov20 (and its homologues) in vivo remains to be established, but there are a number of reasons why this retinol binding molecule may play a critical role in the immune and pathogenesis of filarial nematode infections. The role of retinoids other than in vision is an area considerable interest (2, 9, 10, 32). Filariae concentrate and store retinol in excess of host tissue (28, 30). Retinoids have been shown to be important in development of a type 2 immune response, with vitamin A-deficient mice displaying a tendency toward a type 1 re-
sponse, an effect occurring at the level of antigen-presenting cells as well as at the T-cell level (6, 8). Retinoids influence collagen gene expression, and collagen formation plays a major role in the pathogenesis of onchocerciasis (7). It may be rele-
vant here that a 19-kDa retinol binding protein from O. vol-
vulus is reported to bind ivermectin, the drug currently used for control of the disease (18, 27, 28). The fact that Ov20 appears to have no counterpart in the mammalian host is encouraging in that it could provide a novel therapeutic target for onchocerci-
ciasis therapy.

It is crucial that further studies be done to establish the functional significance of this retinol binding protein, and it is our hope that the cloning of its homologues (Bm20 and Av20) and the availability of two different model systems will help achieve this objective. The greater similarity in both immune reactivity and glycosylation between Av20 and Ov20 suggests that Av20 would be the better model antigen. In addition, important differences in the reactivity patterns of the O. vol-
vulus and B. malayi homologues suggest that differences in the immune recognition of this molecule in the two parasite sys-
tems may be due to differences in glycosylation.

ACKNOWLEDGMENTS

We thank Mark Blaxter, Andrew MacDonald, and Bill Gregory for valuable assistance.

J. Allen is an MRC senior research fellow. N. Nirmalan is a recipient of the Overseas research student award. This work was supported by the INCO-DC programme of the European Commission, the Edna McConnell Foundation, and the MRC.

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Editor: J. M. Mansfield


