**Identification of Chitinase as the Immunodominant Filarial Antigen Recognized by Sera of Vaccinated Rodents**

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Ralf Adam§, Brigitte Kaltmann‡, Werner Rudin¶, Thomas Friedrich¶, Thomas Marti***, and Richard Lucius‡

From the Division of Parasitology, Institute for Zoology, University of Hohenheim, Emil-Wolff-Strasse 34, 70599 Stuttgart, Germany, the Swiss Tropical Institute, Socationstrasse 57, 4051 Basel, Switzerland, the BASF AG, 67056 Ludwigshafen, Germany, **Bernhard-Nocht-Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany

Acanthocheilonema viteae is a parasitic nematode of rodents. We identified the chitinase of A. viteae infective stage larvae (L3) as the main target of the humoral immune response of jirds, which were protected against challenge infection after vaccination with irradiation attenuated L3. The cDNA of the L3 chitinase has been sequenced, and the deduced amino acid sequence shows significant homologies to chitinases of Brugia malayi microfilariae, insects, yeast, bacteria, and Streptomyces sp. The protein has been characterized by monoclonal antibodies and substrate activity gels. The chitinase of L3 may contribute to degrading the nematode cuticle during molting and thus represents a target of protective immune responses in a phase where the parasite is highly vulnerable. In addition, it has been shown that a similar enzyme exists in uterine microfilariae, which probably has a role in casting the egg shell.

Filarial parasites, such as Onchocerca volvulus, Brugia malayi, and Wuchereria bancrofti, the causative agents of river blindness and lymphatic filariases, affect more than 100 million people throughout the tropics (1). Infection of the human host occurs by a bite of an infected arthropod. There is evidence that some individuals develop protective immunity naturally, as they do not acquire filarial infections despite high levels of local transmission (2, 3). The humoral immune response of these patients differentially recognizes antigens of infective third stage larvae (L3) (4), and it is conceivable that vaccination with such antigens could prevent the infection. As the study of protective immunity in human filariae is hampered by the host specificity of the parasites, we adopted the approach to identify immunorelevant antigens of the rodent filaria Acanthocheilonema viteae, a parasite of the jird (Meriones unguiculatus).

For animal models, the vaccination with irradiation-attenuated infective larvae (L3) has been demonstrated to be the most effective way to induce protective immunity (5). Immunization of jirds with irradiation-attenuated L3 of A. viteae induces more than 90% protection against a challenge infection (6). Sera of such vaccinated animals recognize few L3 proteins. We report here on the characterization and the molecular cloning of the immunodominant antigen recognized by the humoral immune response of challenge resistant jirds, a chitinase of A. vitaeae L3. Chitinases were recently described from microfilariae of B. malayi (7). It was supposed that the enzymes might have a role in casting the microfilarial sheath, a modified egg shell. This structure was believed to contain chitin on biochemical grounds (8, 9) and due to its analogy with the chitin-containing egg shell of other nematodes. Interestingly, the egg shell is the only structure of nematodes known to contain chitin. However, recent reports deny the presence of chitin in the microfilarial sheath (see Refs. 10–12) and call the chitin-degrading role of filarial chitinase into question. Our characterization of L3 chitinase (an active, chitin-degrading enzyme) in a filarial stage that is not yet known to contain chitin suggests that the enzyme has additional substrate specificities. Chitinase-like proteins of vertebrates, a class of animals not producing chitin, were described to degrade extracellular matrix under inflammatory or degenerative conditions and to play a role in the process of fertilization (13, 14). The close homology of the L3 chitinase with these molecules suggests that the described enzyme degrades N-acetylglucosamine-containing structures of the parasite during molting and might help the parasite to migrate through the host’s tissues.

**MATERIALS AND METHODS**

Parasites—The life cycle of A. viteae was maintained in jirds (M. unguiculatus) and soft ticks (Ornithodoros moubata) as described earlier (15). Filarial stages were handled in parasite medium (PM) consisting of RPMI 1640 (Life Technologies, Inc.) supplemented with 20 mM Hepes, pH 7.3, 4 mM L-Glu, and 100 units/ml penicillin/streptomycin. Adult filariae were collected by dissection from the subcutaneous tissues of jirds. Uterine MF were obtained by teasing out and mincing the uterus of female worms and collecting the embryonic stages from the PM. Newborn MF were collected from female worms kept in culture for 3 days in PM. Blood MF were isolated from the blood of infected jirds by centrifugation through a Percoll gradient. To isolate vector-derived L3, ticks with 8-weak-old infections were dissected in PM and after 30 min, the emerging L3 were collected. Induced L3 were produced by incubating batches of each 1,000 L3 subcutaneously into jirds, retrieving the larvae from the muscle tissue 3 days postinfection in a Baermann funnel. L4 were obtained by dissecting the jirds 5 days postinfection and culturing the released L3 in PM, where they molted synchronously within 24 h. L4 were obtained from the rodets 13 days postinfection. L3 culture supernatant and molting supernatant were produced by centrifugation through a Percoll gradient. To isolate vector-derived L3, ticks with 8-weak-old infections were dissected in PM and after 30 min, the emerging L3 were collected. Induced L3 were produced by incubating batches of each 1,000 L3 subcutaneously into jirds, retrieving the larvae from the muscle tissue 3 days postinfection in a Baermann funnel. L4 were obtained by dissecting the jirds 5 days postinfection and culturing the released L3 in PM, where they molted synchronously within 24 h. L4 were obtained from the rodets 13 days postinfection. L3 culture supernatant and molting supernatant were produced by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) U14638.

§ Received a scholarship from the “Studienstiftung des deutschen Volkes.” To whom correspondence should be addressed: Inst. for Molecular Parasitology, Humboldt University Berlin, Invalidenstr. 43, D-10115 Berlin, Germany. Tel.: 49-30-2895255; Fax: 49-30-2895251; E-mail: un101@geniius.emnet.de

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Filarial Chitinases

culturing batches of 1,000 vector-derived L3 or 1,000 induced L3, respectively, in 500 μl of PM for 24 h. The supernatants were cleared by centrifugation and filtering through a 0.22-μm membrane filter.

Sera and Monodonal Antibodies (mAbs)—Sera were collected from jirds vaccinated with irradiation-attenuated L3 and challenged with vector-derived L3 as described previously (6). The worm burden of the vaccinated jirds was >10% of the worm burden of control jirds. In 3 min of continuous incubation, the production of mAb 24-4 (IgG1, κ L chains) against irradiated L3, 6-week-old BALB/c mice were immunized twice in biweekly intervals by subcutaneous injection of 50 irradiated L3 of A. viteae. To produce mAb 2H2 (IgM, κ L chains) against molting supernatant, animals were immunized thrice in biweekly intervals with 500 μl of molting supernatant containing 1% thioglycolic acid and blotted onto polyvinylidine difluoride membrane (Immobilon, Millipore, Bedford, MA). Alternatively, the proteins were solubilized in sample buffer containing 100 mM dithiothreitol instead of 2-mercaptoethanol, 0.1% SDS, 5% (v/v) glycerol in 63 mM Tris-HCl, pH 6.8, to which were added traces of bromphenol blue and protease inhibitors. 1% (v/v) of a stock solution of 100 μl of STP (sodium metaperiodate, Tween 80, pluronic L101) was added (see Ref. 6). 5 days prior to the fusion, the animals were boosted with a further dose of irradiated L3 or molting fluid, respectively, given intraperitoneally. Spleen cells of these animals were fused with the BALB/c myeloma X63/Ag8 and selected according to standard methods (16). A mAb against a genus-specific epitope of O. volvulus (IgG1) served as control antibody.

Immunochemical Characterization of Chitinase—Filarial proteins were solubilized in sample buffer consisting of 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol in 63 mM Tris-HCl, pH 6.8, to which were added traces of bromphenol blue and protease inhibitors. 1% (v/v) of a stock solution of 100 μl of a 1:10 dilution of phenylmethylsulfonyl fluoride (Serva, Heidelberg, Germany) in methanol. Alternatively, the proteins were solubilized in sample buffer containing 100 mM dithiothreitol instead of 2-mercaptoethanol as a reducing reagent. The proteins were separated by SDS-PAGE (17) and blotted onto nitrocellulose membranes (18). The sheets were reacted with unidiluted hybridoma supernatant or appropriate dilutions of jird sera. Immune complexes were detected with horseradish-conjugated anti mouse IgG1+IgM (Tago, Burlingame, CA). For the indirect fluorescent antibody test (IFAT), L3 were fixed overnight at 4°C in 1% (v/v) formaldehyde in PBS. PBS washed 120 μl of PM for 24 h. The supernatants were cleared by centrifugation and filtering through a 0.22-μm membrane filter and probed with the BALB/c myeloma X63/Ag8 and selected according to standard methods (16). A mAb against a genus-specific epitope of O. volvulus (IgG1) served as control antibody.

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that can be reduced by high concentrations of sulfhydryl compounds.

As mAb 24-4 was directed against an epitope stable under a variety of conditions, it served to localize the filarial chitinase in L3. Immunogold staining of ultrathin sections of L3 with mAb 24-4 revealed the presence of *A. viteae* chitinase in the cellular cytoplasm and in the lumen of the glandular oesophagus (Fig. 4A). No chitinase could be detected in muscle, cuticle, or on the outermost surface of L3. Neither live L3 nor formaldehyde-fixed L3 studied under a variety of conditions by IFAT carried detectable amounts of chitinase on the surface.

Characterization of L3 Chitinase cDNA—Degenerate primers P1 and P2 derived from the N-terminal sequence of the *A. viteae* L3 chitinase and a region conserved within the chitinases of *B. malayi* and *W. bancrofti* (7, 23) were used to amplify a 580-base pair cDNA sequence by PCR with a L3 cDNA library of *A. viteae*. The amplification product was sequenced, and 88% of the nucleotides were identical with the corresponding sequence of the published *B. malayi* MF chitinase. This indicated that a part of a chitinase gene of *A. viteae* had been amplified. The sequence was labeled with digoxigenin and used as a probe to isolate clones from the L3 library. Screening of 8 x 10^5 phage plaques yielded 13 positive clones after three rounds of screening. All inserts had the same length as shown by PCR with vector-specific primers corresponding to the T3 and T7 promoter regions. One full-length clone was sequenced in both strands using sequence-specific primers P1–P6 and vector-specific primers for the T3 and T7 promoter regions.

The obtained sequence was 1,670 base pairs long and contained an open reading frame coding for 520 amino acids with a theoretical molecular mass of 58 kDa (Fig. 2). The N-terminus consisted of amino acids identical with the corresponding sequence of the published *B. malayi* MF chitinase. The 69% nucleotide identity over the whole sequence to the MF chitinase of *B. malayi* (7) and weaker similarities to chitinases of *W. bancrofti* (23), insects, bacteria, *Streptomyces* sp., fungi, and plants (30–32). The domain structure was similar to the one of *B. malayi* MF
chitinase. The N-terminal signal sequence is followed by the well-conserved catalytic domain, spanning amino acids 18–370 (31–33). The third domain is less conserved and contains 35% Ser and Thr residues. It could be a target of extensive O-glycosylation (7, 31). This region comprises four imperfect repeats of 14 amino acids in length between positions 370 and 440 of the mature protein. The carboxyl-terminal end of the protein is closely related in structure to the B. malayi and insect chitinases as the 6 Cys residues are perfectly matched, suggesting that they could be involved in disulfide bridges. However, these regions are different from the chitin-binding domains described for yeast and class III plant chitinases.

To study the presence of chitinase mRNA, cDNA from male worms, female worms, blood MF, L3, and L4 was amplified with degenerate primers P1 and P2. In all stages, except male worms, specific amplification products were obtained, indicating that chitinase mRNA is present in most filarial stages (not shown).

Characterization of Uterine MF Chitinase—Studies with the mAbs against L3 chitinase revealed that uterine MF bear epitopes common to L3 chitinases. mAb 2H2 (binding to the 68- and 205-kDa proteins) is underlined. The N-terminal amino acid sequence derived from the purified 205- and 68-kDa proteins is boxed and shadowed, and the amino acids resulting from the 205-kDa protein are in italics. Potential myristilation sites are indicated by an inversed Gly residue. The attachment site for glycosaminoglycan (amino acids 234–237) is shaded. One potential N-glycosilation site is doubly underlined. Conserved Cys residues within the carboxyl-terminal domain are indicated in boldface. The four repeated amino acid sequences in the C-terminal domain are boxed and shadowed.
band of female worms co-migrated exactly with the band recognized by mAb 2H2.

Immunogold staining of ultrathin sections of female worms with mAbs 24-4 revealed that chitinase was localized in the epidermis and in the laminated layer of the cuticle of most MF present in the uteri (Fig. 4B). Only a small proportion of the MF carried the antigen on the outermost surface. The egg shell itself and other compartments of the MF did not contain detectable amounts of chitinase. To study the presence of chitinase on the surface of uterine MF, we performed IFAT with mAbs 24-4 and 2H2 using cryostat sections and preparations of uterine content of female A. viteae, where both mAbs immunostained exactly the same structures. IFAT analysis revealed that the target epitopes were accessible to the mAbs on nearly mature uterine MF, where chitinase was evenly distributed on the surface. Most of these Ag-bearing MF were still within the egg shell (Fig. 5, A and B). In contrast, no more chitinase was present on the surface of fully mature, hatched uterine MF, which are more slender than the nearly mature forms and have pointed heads. However, MF, which had degenerated within the female worms around the time of molting and were stumpy, immotile, and sometimes broken, carried detectable amounts of chitinase on their surface. An IFAT study of newborn MF revealed that such degenerated MF bearing chitinase on their surface represented around 10% of the MF production released by in vitro cultured female A. viteae (Fig. 5, C and D).

**DISCUSSION**

The most effective way to induce protective immunity against filarial infections in experimental animals is the immunization with irradiation-attenuated L3 (see Refs. 5 and 6). Our study reveals that L3 chitinase is the immunodominant antigen recognized by the humoral immune response of jirds vaccinated against *A. viteae* infection. This protein has several properties of a vaccine candidate antigen; in particular it is exported during the initial phase of the infection and during molting of L3, which are phases of key importance for the attrition of filarial larvae (34, 35). Furthermore, L3 culture supernatants and molting fluid, which both contain chitinase, induce protection against a challenge infection with *A. viteae* in jirds (6).2 The findings of Freedman et al. (3), describing a 43-kDa chitinase-like filarial protein to be recognized by the sera of persons resistant against infection with the lymphatic filaria *W. bancrofti* (23), support the notion of chitinase being a protein with protective properties. Immunization studies in our animal model will evaluate whether immune responses against L3 chitinase can prevent an infection with *A. viteae*.

Chitinases are enzymes that hydrolyze chitin (poly-β-(1-4)-linked GlcNAc). Well known examples of chitinases are the enzymes of insects (*Manduca*), which have a role in degrading the chitinous exoskeleton during the molt and the chitin-degrading enzymes of fungi and streptomycetes (30–32).

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2 R. Adam, B. Kaltmann, W. Rudin, T. Friedrich, T. Marti, and R. Lucius, unpublished results.

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![Image](image-url)
However, chitinases can cleave substrates other than chitin and can have activities as trans-glycosidases (36). The role of chitinase in L3 is unclear, and the fact that the enzyme is released by the L3 during different phases suggests that it might have a broad substrate specificity. First, L3 chitinase is exported into the culture medium immediately after the larvae are cultured under vertebrate conditions. This time point corresponds to the early infection where L3 migrate through host tissues (37). An enzyme exported during this phase could help to degrade host tissues, provided that it has an appropriate substrate specificity. Interestingly, chitinase-like molecules were recently described from the cartilago and the oviduct of vertebrates (13, 14, 38–40), which are supposed to contribute to the degradation of extracellular matrix under inflammatory or degenerative conditions or to fertilization of vertebrates. Second, L3 chitinase is produced during molting, a phase where the nematodes’ cuticle is reorganized and finally cast. It is not clear whether chitin is a target substrate during this phase, as the only stage of nematodes that has been demonstrated to contain chitin is the egg (41–42). However, recent studies described chitotriosides in the cuticle of adult Haemonchus contortus, which is indicative for chitin being a structural component of the nematode cuticle (43). Therefore, further studies have to show whether the target substrate of L3 chitinase is chitin or whether other substrates are converted as \( \beta-(1\rightarrow4) \)-linked N-acetylglucosamine oligomers, which were shown to occur in the filarial cuticle by lectin binding studies or enzymatic studies (19, 43). Irrespective of the substrate, the localization of the enzyme and the timing of release suggest that L3 chitinase is involved in the process of molting. The source of released L3 chitinase are the pharyngeal glands, where the enzyme was localized by immunoelectron microscopy. Pharyngeal gland products, which are exported through the buccal cavity, were described to have a role in molting in other nematodes (44) and are released around the time of molting by L3 of O. volvulus (45). Molting products of nematodes are so far poorly described and the only pharyngeal gland component described on the molecular level is a metalloprotease of H. contortus (46). Recent data indicate that the production of L3 chitinase is regulated by steroid hormones, which is an analogy to the tightly regulated expression of insect chitinases during molting (30).

Our study suggests that the chitinase of uterine MF has a role in cleaving chitinous structures of the eggshell of A. viteae. Chitin was detected in the eggs of intestinal helminths Ascaris suum and Heligmosomoides polygyrus (41, 47) and it was described to be a component of the egg shell of the filariae Onchocerca gibsoni and O. volvulus (42). Chitinase is considered as a relevant factor for the casting of the egg shell of A. suum (47). The strict coincidence of the occurrence of chitinase on the surface of uterine MF of A. viteae and the time point of molting suggests that A. viteae chitinase contributes to hatching. It is conceivable that chitinase detected within the cuticle of immature uterine MF represents a storage form, which is transported to the MF surface prior to the hatching. The lack of chitinase in blood MF of A. viteae suggests that MF of this filarial species remain surrounded by the modified egg shell, which is not cast until the blood MF have entered the arthropod host (48).

The cDNA of the A. vitaeae L3 chitinase shows 69% nucleotide identity with the cDNA of B. malayi MF chitinase (7). The chitinase of the tobacco hornworm Manduca sexta (30) is also closely related to the filarial chitinases as shown by the similarity of the enzyme domain structure and by the amino acid composition (28% of the amino acids are identical, and about 70% are similar), whereas the described chitinases of streptomycetes, bacteria, and fungi are less related to our molecule.

![Fig. 5. Localization of A. vitaeae chitinase in intrauterine MF by IFAT with mAb 24-4 (left panels) and corresponding light microscope photographs (right panels).](image)
The sequence of the chitinase-like 43-kDa molecule of W. bancrofti (23), which is derived from genomic DNA, has two small regions of homology in common with A. viteae L3 chitinase and B. malayi MF-chitinase but is otherwise relatively distinct. The homology between these filarial chitinases is highest (86% nucleotide identity) in the N-terminal signal sequence and the adjacent 370-amino acid region, the catalytic domain. The catalytic domain of A. viteae L3 chitinase is identical to the one of uterine MF of A. viteae and very closely related to the one of uterine MF of O. volvulus.2 Downstream follows a Ser/Thr-rich domain, which encompasses three nearly perfect repetitions of 14 amino acids in the B. malayi gene, whereas this region shows four imperfect repetitions of each 14 amino acids in the A. viteae gene. The Ser/Thr-rich domain is 21 amino acids longer in the A. viteae cDNA. The carboxyl-terminal Cys-rich domain shows similarities between the filarial chitinases and the enzyme of M. sexta. The 6 Cys residues are perfectly matched, indicating a conserved function of this domain, which is potentially important for intra- and intermolecular bridging. The fact that the open reading frame of the L3 chitinase cDNA codes for a protein with a theoretical molecular mass of 58 kDa suggests that the protein backbone is posttranslationally modified. The discrepancy between theoretical and actual molecular mass is probably due to extensive O-glycosylation of the Ser/Thr-rich domain and the very acidic composition of this region, which could be responsible for an anomaly in the migration in SDS gels (7, 31). Deglycosylation of native A. vitaeae L3 chitinase using O-glycanase resulted in a shift of molecular weight. Expression of the cDNA in Escherichia coli yielded a 58-kDa protein, which hydrolyzed chitin, indicating that the activity of the enzyme is not dependent on glycosylation.2 Our experiments show that the 205-kDa band of L3 is a multimeric form of chitinase, derived from 68-kDa monomers by disulfide bridging. Formation of multimers was associated with an alteration of the antigenicity and of the chitinase activity, since mAb 24-4 did not bind to the 68 kDa band and the 205 kDa protein was shown to be less active in substrate gels.

The presence of chitinase in two distinct stages of the parasite's life cycle and the stage specific localization of the enzyme suggest that the expression of chitinases of L3 and MF is specifically regulated. Furthermore, the differences of molecular weight between L3 chitinase and MF chitinase show a specific structure of the molecules. Sequencing of the C terminus of the cDNAs of both stages revealed sequence variation,2 being suggestive for the presence of several chitinase genes. However, it is also possible that stage-specific differential splicing or differential posttranslational modifications contribute to the observed differences between L3 chitinase and MF chitinase of A. viteae. It will be interesting to study which specific properties are related to the timing of expression, the localization of the enzyme within the parasites, and the substrate specificity of chitinases of various stages and species of filarial parasites.

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