

Identification, characterization and expression of *Toxocara canis* nematode polyprotein allergen TBA-1

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Abstract

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We have cloned the cDNA of TBA-1, the Nematode polyprotein allergen (NPA) of Toxocara canis and found it to be most similar to ABA-1, the Ascaris NPA, on the basis of amino acid sequence. We could study the antigenic properties of an E-coli synthesized fusion protein prepared with the cloned gene since no glycosylation site was expected from the deduced amino acid sequence. Although no IgE responses to TBA-1 were detected, recombinant TBA-1 was differently recognized by serum IgG antibodies when the recombinant TBA-1 was directly adsorbed vs when immobilized via a streptavidin linkage on polystyrene microtitre wells. One group of sera recognized TBA-1 directly immobilized while the second only recognized TBA-1 immobilized via streptavidin linkage. The former were from rodents immunized with a Toxocara sp. adult worm extract while the latter were obtained from rodents infected with T. canis larva or immunized with a Anisakis simplex L3 larval extract. These observations suggest that the two in vivo forms of TBA-1 are expressed, but during different stages of the parasite's life cycle.

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Nematode antigens responsible for Type 1 hypersensitivity have been identified using biochemical methods and recombinant DNA technology ([Christie, Dunbar & Kennedy 1993](#), [Paxton, Yazdanbakhsh, Kurniawan et al. 1993](#)). *Ascaris* body fluid allergen-1 (ABA-1) was the first well-characterized *Ascaris* protein and is a major component of the pseudocoelomic fluid of *Ascaris* ([Kennedy, Qureshi, Fraser et al. 1989](#), [Christie et al. 1993](#), [Spence, Moore, Brass et al. 1993](#)). ABA-1 homologues are found in numerous nematodes and are collectively called 'nematode polypeptide allergen' (NPA); ([McReynolds, Kennedy & Selkirk 1993](#)). NPA is approximately a 15-kDa antigen and is abundant in both somatic and excretory/secretory products (ES) of the parasites ([Christie et al. 1993](#), [Paxton et al. 1993](#), [McReynolds et al. 1993](#), [Kumari, Lillibridge, Baker et al. 1994](#)). NPAs are synthesized as large polypeptides that are subsequently proteolytically cleaved to 15-kDa polypeptide units ([McReynolds et al. 1993](#), [Paxton et al. 1993](#)). The cDNA indicates that the NPA precursor consists of repeat units, each of which encodes a 15-kDa protein. In most cases, there is little variation between the amino acid sequence of the repeat units within a given array except in a case of *Dictyocaulus viviparus* ([Britton, Moore, Gilleard et al. 1995](#)). TBA-1 is the NPA of *Toxocara canis*, whose N-terminal amino acid sequence reveals a high degree of similarity to ABA-1, the *Ascaris* NPA ([Christie et al. 1993](#)). In this study, we determined a part of the sequence of the TBA-1 precursor cDNA which contains at least one complete repeat unit. The recombinant form of TBA-1 was synthesized using a bacterial expression system and the immunogenic property of the fusion protein was tested by solid phase immunoassays.

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cDNA Library

A *T. canis*, adult worm cDNA Unizap II expression library was constructed in accordance with the manufacturer's procedure (Stratagene, La Jolla, CA, USA). The mRNA was prepared from an adult female worm collected from a young dog. A *T. canis* larval cDNA expression library was kindly given by Heska Corporation, Fort Collins, CO, USA.

Immune sera

Sera from immunized or infected animals were obtained as described below and kept at -70°C until use.

(1) The sera of mice infected with *T. canis* or *T. cati* were obtained after inoculation of six week-old ddy male mice *per os* with 2000 *T. canis* or *T. cati* embryonated eggs. The sera were collected 12 weeks after infection and pooled.

(2) The sera of rabbits immunized with *T. canis* or *T. cati* adult worm extract were obtained from four Japanese white female rabbits that had been immunized with an extract of *T. canis* (TCNEX) or *T. cati* adult worm (TCTEX) in the presence of complete Freund's adjuvant. The immune sera were collected bi-weekly for six weeks following immunization.

(3) The sera of rabbits infected with *T. canis* were obtained from four Japanese white female rabbits that had been inoculated *per os* with 2000 *T. canis* embryonated eggs. The sera were collected bi-weekly for eight weeks following infection.

(4) Sera were obtained from rats immunized with various nematode antigens. These included sera from four six month old Wistar strain female rats that had been immunized twice with an extract of TCNEX, TCTEX or *A. suum* (ASSEX) adult or an extract of *Anisakis simplex* L3 (ANSEX) larvae. Complete Freund's adjuvant was used in all cases and the sera were collected four weeks after the first immunization.

Sera from non-immunized and uninfected rabbits, rats and mice were used as controls.

Immunoscreening

The adult and the larval expression cDNA libraries were immunoscreened with the serum from mice infected with *T. canis* (diluted 1:5) and/or the serum from a rabbit immunized with TCNEX (diluted 1:10 000) in accordance with Picoblue™ immunoscreening kit protocol (Stratagene). The cross-reactivity of each selected clone was tested using a dot-blot ELISA with the sera from mice infected with *T. canis* or *T. cati* or the sera from rabbits immunized with TCNEX or TCTEX.

Sequencing of *Toxocara canis* cDNAs

Clones isolated after immunoscreening were *in vivo* excised and the double stranded inserts were sequenced using the dideoxynucleotide termination method with the help of T3, T7 and synthetic TBA-1 specific primers. Cycle sequencing reactions were performed with ABI PRISM™ sequence kit (Perkin Elmer, Foster City, CA, USA).

Subcloning of the TBA-1 insert and expression and purification of the fusion protein

The TBA-1 insert was amplified by PCR using the T3/T7 primer set. The PCR product was digested with *Bam*HI and *Kpn*I and subcloned into PinPoint Xa-2 vector (Promega, Madison, WI, USA). The vector was transformed into XLI MRF' competent cells. Mini and large scale cell culture, as well as induction and purification of the fusion proteins, was, performed in accordance with the manufacturer's procedure. The fusion protein contains a biotin moiety enabling it to be captured by avidin or streptavidin.

Solid phase immunoassays

1. Western blot

Lysates of isopropyl-1-thio-β-D-galactoside (IPTG)-induced *E. coli* carrying the expression construct, were analysed in Western blots in accordance with the PinPoint™ Purification System technical manual. In brief, IPTG-induced *E. coli* cells were pelleted, lysed and denatured with 1X sample buffer (12.5% stacking gel 4X buffer, 1% SDS, 2.5% ββ-mercaptoethanol, 10% glycerol, 0.00125% bromophenol blue) followed by incubation for 5 min at 95°C. Proteins were electrophoresed in 12.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Japan, Tokyo, Japan). The membranes were completely dried and then incubated for one h at RT with a 1:100 dilution of sera from rabbits immunized with TCNEX or TCTEX or the sera from rabbits infected with *T. canis* in 1% BSA-PBST (1% bovine serum albumin, 0.067 M PBS, 0.05% Tween 20). The membranes were sequentially incubated with a 1:500 dilution of anti-rabbit IgG (H+L) conjugated to alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) for 30 min at RT. The membranes were washed three times with PBS between the consecutive incubation steps. The colour reaction was developed with 0.03% nitroblue tetrazolium and 0.015% 5-bromo-4-chloro-3-indolyl phosphate (w/v) in the substrate buffer for

alkaline phosphatase (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl pH 9.5).

2.1 Detection of rabbit IgG by ELISA

The wells of a polystyrene plate (TC 96F Microwell, NUNC, Roskilde, Denmark) were incubated with 100 µl of 10 µg/ml avidin in PBS at 4°C overnight. The solution in the wells was then aspirated and the plate was washed with PBST and stored at 4°C until further use. To each well of the plate, 100 µl of TBA-1 fusion protein (125 ng/ml) in 1% BSA-PBST, was added and the plate was incubated at 37°C for one h. The plate was then washed with PBST and each well was filled with 100 µl of a 1:100 dilution of the sera from rabbits immunized with TCNEX or TCTEX or the sera from rabbits infected with *T. canis* in 1% BSA-PBST. After incubation at 37°C for one h, the plate was again washed, and 100 µl of a 1:200 dilution of peroxidase anti-rabbit IgG (H + L); (Vector Laboratories) in 1% BSA-PBST, was added to each well. After incubation at 37°C for one h with this conjugate, the wells were washed and 100 µl of a 0.6 mg/ml solution of 2',2'-azino-di(3-ethylbenzothiazoline sulphonate); (ABTS) in ABTS substrate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄ and 0.03% H₂O₂) was added. The enzymatic reaction was stopped with 20 µl of 1.25% sodium fluoride and the optical densities were measured at 405 nm using a microplate reader.

2.2 Detection of rat IgG by ELISA

TBA-1 was immobilized direct adsorption or via streptavidin linkage to microtitre wells. Two-fold serial dilutions from 1:10 to 1:1,280 were tested against TBA-1 immobilized by each method. Commercial streptavidin-precoated plates (Combiplate 8 Streptavidin Coated; Labsystems, Helsinki) were used instead of manually coated plates (see above). Other aspects of the assay followed the same protocol used for ELISA detection of rabbit antibody except peroxidase anti-rat IgG (Chemicon International Inc., Temecula, CA, USA) was employed as the secondary antibody (1:10 000 dilution).

RESULTS

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Immunoscreening

When the larval cDNA library was immunoscreened with pooled serum from mice infected with *T. canis* or serum from a rabbit immunized with TCNEX, 40 positive clones were obtained from 200 000 plaques in each case. No clones were recovered from the adult library when at least 1 000 000 plaques were immunoscreened using the pooled serum from mice infected with *T. canis*. Furthermore, no positive clones were detected in either the two cDNA libraries when the control sera were used. Ten positive clones were selected in each case and tested by dot-blot ELISA. The pooled sera from mice infected with *T. cati* also recognized nine of ten positive clones detected with the pooled serum from mice infected with *T. canis*. The remaining clone was exclusively recognized by the pooled serum from mice infected with *T. canis*. All ten clones detected with the serum from a rabbit immunized with TCNEX also tested positive with the sera from rabbits immunized with TCTEX.

Sequence analysis of *T. canis* cDNA clones

The cDNA sequences of the isolated clones were compared to sequences in the Plot data base. We found four kinds of clones (data not shown). TBA-1 was found with the rabbit immunized serum with TCNEX. We designated TBA-1 clones A1-A10. Two TBA-1 clones (A1 and A6) were selected for complete sequence analysis because fusion-proteins expressing these cDNA were available in the largest amount.

Our TBA-1 sequence data, especially that obtained with clones A1 and A6, reveal that the TBA-1 cDNA consisted of 399 bp repeat units organized in a head to tail array. The tandem repeat structure was also confirmed by PCRs with TBA-1 cDNA specific primers (data not shown). [Figure 1](#) gives the cDNA sequence of A1. A1 contained one tandem repeat unit. The coding region of A6 contained one 87 bp-truncated (312 bp) and one complete (399 bp) TBA-1 coding units (data not shown). We performed PCRs using cDNA of a *T. canis* adult worm and A1-10 clones with TBA-1 specific primer sets in order to investigate TBA-1 complete cDNA configuration. We found the cDNA has at least two repeat TBA-1 coding units (data not shown). The predicted cDNA structure was shown in [Figure 2](#).

Figure 1. The nucleotide sequence of the A1 cDNA sequence containing a complete coding region at its predicted amino acid sequence. The coding region consists of one repeat unit of TBA-1 followed by a carboxyl terminal region (underlined). *: stop codon.

Figure 2. Predicted configuration of complete TBA-1 cDNA. 5' and 3' UTRs: untranslated regions of 5' and 3' ends respectively. SLN1: spliced leader sequence.

[Figure 3](#) compares the predicted amino acid sequences of the different TBA-1 clones with that of the published ABA-1 sequence. The consensus deduced amino acid sequences of the TBA-1 cDNA was most similar to that of ABA-1. The sequence of TBA-1 from clone A1 consisted of 133 amino acids and the alignments indicated 86.5% identity and 93.2% similarity to ABA-1. The A6 clone encoded a truncated (A6-1) and a complete (A6-2) TBA-1. A1 and A6-1 encode a predicted proteolytic cleavage site (Arg-Arg-Arg-Arg) with processing proteinases of the subtilisin family while A6-2 did not ([McReynolds et al. 1993](#)). Small variations in the deduced amino acid sequence between A1 and A6 were also found. Neither TBA-1 nor ABA-1 had any conventional N-linked glycosylation sites (Asn-X-Ser or Asn-X-Thr) ([Lodish, Baltimore, Berk et al. 1995](#)).

Figure 3. Alignments of TBA-1 and ABA-1 amino acid sequences. Bold and italicized characters indicate identical amino acids between TBA-1 and ABA-1 and between TBA-1 clones respectively. Underlined characters indicate the cluster of four basic amino acids at which proteinases of the subtilisin family are presumed to cleave the sequence. A1: The TBA-1 repeat encoded by A1. A6-1: The first TBA-1 repeat encoded by A-6. The 5' end of TBA-1 gene is truncated. A6-2: the second TBA-1 repeat unit encoded by A6. ABA-1: ABA-1 unit.

Subcloning of the TBA-1 inserts and expression and purification of the fusion protein

The clone A1 was chosen for use in fusion protein expression because it contains one unit of TBA-1 repeat. *E. coli* expressed enough TBA-1 fusion protein to be detectable by Western blotting (data not shown).

Immunological examinations

[Table 1](#) summarizes the results of serological studies in rabbits. Western blots detected IgG antibodies to TBA-1 in all immunized rabbits and one infected rabbit. A transient TBA-1 specific IgG response was also detected by ELISA in two of the four infected rabbits (the 4th and 6th week respectively) but no TBA-1 specific IgG response was detected in the immunized rabbits or in the control. Rapid TCNEX specific IgG responses were observed in all the infected and immunized rabbits (data not shown).

Table 1. Summary of the results of TBA-1 specific IgG Western blot and ELISA analyses of rabbits

[Figure 4](#) shows that specific IgG to directly immobilized TBA-1 was only detected in the serum from a rat immunized with TCNEX while TBA-1 immobilized via a streptavidin linkage was only detected in the serum from a rat immunized with ANSEX.

Figure 4. Detection of rat IgG antibodies to adsorbed TBA-1 (b) or TBA-1 immobilized via streptavidin bridge (a). □ TCNEX; ■ ANSEX; △ Control.

DISCUSSION

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NPAs have been found among several parasitic nematodes and show no similarity to any other proteins ([McReynolds et al. 1993](#)). TBA-1 and ABA-1 share common epitopes and the N-terminal amino acid sequence analysis reveals a high degree of similarity ([Christie et al. 1993](#)). ABA-1 was reported both as an ES and a body extract component. However, unlike other NPAs, [Kennedy et al. \(1989\)](#) reported that TBA-1 exists only in *T. canis* body extract but not in the ES. They also showed that anti-TBA-1 antibodies were not found in animals infected with *T. canis* for prolonged period. Nevertheless, we found a transient TBA-1 IgG antibody response during the infection (fourth and sixth week). The transient nature of this IgG response may explain the failure of [Kennedy et al. \(1989\)](#) to make a similar observation.

TBA-1 specific rat IgE responses were also examined using a TBA-1 specific IgE ELISA (both direct adsorption and via streptavidin linkage) with the sera described in the materials and methods after preabsorption of IgG with Protein G. TBA-1 specific rabbit IgE responses were tested by passive cutaneous anaphylaxis to TBA-1 with the sera described in the materials and methods. No IgE responses were found in either case (data not shown). Since NPA specific IgE responses are MHC-restricted ([Christie *et al.* 1993](#)) it is unlikely that this could explain our failure to detect IgE antibodies since we detected IgG antibodies; differences in assay sensitivity are a more likely explanation.

Preservation of the native epitopes of the TBA-1 recombinant antigen is more likely when TBA-1 is immobilized via a streptavidin bridge. By contrast, direct adsorption can cause conformational changes, which can alter antigenic behavior. The differences in antigenicity that we observed when TBA-1 was immobilized by different methods may reflect such changes in conformation. The phenomenon of adsorption-induced conformational change is well-recognized and the topic reviewed elsewhere ([Butler 1992](#), [Butler, Navaro & Sun 1997](#)).

Recently [Kato & Komatsu \(1996\)](#) reported an anti-bacterial peptide in the body fluid of *A. suum* and its homology (in its N-terminal amino acid sequence) to ABA-1. They concluded that the similarity of denatured ABA-1 might be responsible for the anti-bacterial activity. However, purified ABA-1 failed to show anti-bacterial activity. Alternatively, two different forms of ABA-1 could also explain such a result. In our experiments, TBA-1 immobilized via a streptavidin linkage was recognized only by the sera of rodents infected or immunized with larvae (*T. canis* and *A. simplex*) while directly immobilized TBA-1 was mainly recognized by the sera of rodents immunized with *Toxocara* adult worm extracts. Thus, two different forms of TBA-1 may be expressed in a stage specific manner.

The major difference between the native and the recombinant nematode antigens that are synthesized by *E. coli* is associated with the posttranslational step. *E. coli* is not able to add oligosaccharide chains to its fusion peptides and N-linked glycosylation plays a critical role in host antigenic recognition ([Allen, Lawrence & Maizels 1995](#)). Unlike gp15/400 an NPA of *Brugia pahangi* and *B. malayi*, the deduced amino acid sequence of TBA-1 contains no predicted N-glycosylation site ([Tweedie, Paxton, Ingram *et al.* 1993](#)). This suggests that for TBA-1, the recombinant form of TBA-1 may be more similar to the natural form than in case of gp15/400. For this reason, the recombinant form of TBA-1 may be more useful than gp15/400 for the development of immunodiagnostic assays based on the use of NPAs.

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