Onchocerca volvulus Larval Antigen, OvB20, Induces Partial Protection in a Rodent Model of Onchocerciasis

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OvB20 is an antigen of *Onchocerca volvulus* preferentially recognized by sera from cattle vaccinated with irradiated infective larvae of *Onchocerca lienalis*. Antibodies raised against the recombinant protein were used to characterize the expression of the native protein in different developmental stages of *O. volvulus* and the rodent filaria *Acanthocheilonema viteae*. In *O. volvulus*, antibodies reacted to a polypeptide of 42 kDa in microfilariae and with proteins of 52 and 65 kDa in third-stage larvae. No products were detected in adult stages. Immunogold electron microscopy localized the native protein to discrete patches of the hypodermis and cuticle of infective larvae. Characterization of a homologous protein in *A. viteae* confirmed the stage-specific expression in infective larvae of the 65-kDa protein, which was secreted during *in vitro* culture. Vaccination of rodents against *A. viteae* with a B20-maltose-binding-protein fusion protein resulted in a 49 to 60% reduction in adult worm recoveries with a corresponding 97% reduction in microfilaremia.

Onchocerciasis remains a major public health problem throughout Africa and Central and South America, where millions of people develop progressive and debilitating disease (2). Evidence for the existence of immunity to *Onchocerca volvulus* infection in humans resides in a minority of putative immune individuals, who appear to remain free from infection despite ongoing exposure to the organism (9, 26). In contrast to persons with patent infection, the immune response of these putative immune individuals is characterized by proliferative Th1 cell responses to parasite antigens, suggesting that T-cell-mediated protective responses are responsible for controlling infection (8). Antigen recognition directed preferentially to larval stages is also a feature of the immune response of these people (18). This is consistent with experiments in animal models of filariasis that have identified developing larvae as targets of protective immunity (7). These observations, together with the induction of protective immunity in animal models of onchocerciasis (12, 22), have provided the basis to explore the development of vaccines as part of the long-term control of onchocerciasis.

Induction of protective immunity in animal models of filariasis has been achieved most consistently by exposure to irradiated infective larvae (1a, 12, 15, 19, 22, 27, 29). The mechanism by which irradiation facilitates enhanced immunity has not been characterized. One possibility is that attenuation of larval development alters antigen expression either structurally or temporally, resulting in increased immunogenicity. We have attempted to define antigens associated with exposure to irradiated larvae in the natural host-parasite system of *Onchocerca lienalis* in cattle with a view to identifying recombinant proteins with potential as vaccines against human onchocerciasis.

Previously, we have cloned and sequenced a cDNA encoding an *O. volvulus* antigen, termed OvB20, which was preferentially recognized by antibodies from cattle immunized with irradiated *O. lienalis* third-stage larvae (L3 larvae) (1). OvB20 is the

product of a single-copy gene which is conserved among filarial nematodes but appears to be absent from a range of other species. *In situ* hybridization revealed that transcription is confined to larval stages, with initiation in embryos and maximal production during L2 to L4 development. Although the nucleotide and predicted amino acid sequences demonstrated no overall homologies to known genes, a central block of sequence with similarities to the acute-phase reactant C-reactive protein has been described elsewhere (1).

In the present study, we have used antibodies raised against the carboxy terminus of the recombinant antigen to characterize the native parasite product and identify its stage specificity and ultrastructural localization. To investigate the potential of OvB20 to induce protective immunity, the cDNA was subcloned into the pMALcR1 expression system and the fused protein was used in vaccination experiments to test its efficacy against developing larvae and microfilariae in animal models.

MATERIALS AND METHODS

Parasites. Skin microfilariae of *O. lienalis* were obtained from samples of umbilical skin collected from naturally infected British cattle at slaughter. The parasites were recovered under aseptic conditions, as described previously (3, 6). L3 larvae of *O. lienalis* were produced in *Simulium ornatum* sensu lato. Blackflies, obtained by eclosion of pupae in the laboratory, were infected by intrathoracic injection of microfilariae (4). Cryopreserved L3 larvae of *O. volvulus* were obtained from Milan Trpis and Sara Lustigman under an Onchocerciasis Resources Project from the Edna McConnell Clark Foundation. The parasites were thawed in a circulating water bath at 37°C and washed in RPMI 1640 culture medium. Adult worms and microfilariae of *O. volvulus* were recovered from nodules excised from patients attending a clinic at the Medical Research Laboratories in Bo, Sierra Leone (as part of a clinical management program).

The related filaria *Acanthocheilonema viteae* was obtained from a laboratory-adapted cycle maintained in Mongolian jirds (*Meriones unguiculatus*) and argasid ticks (*Ornithodoros tartakovskyi*) (28). L3 larvae were obtained from infected ticks by dissection in RPMI 1640 medium. Microfilariae were filtered from the blood of patently infected jirds, washed in RPMI 1640 medium at 37°C for 30 min, and passed through a PD-10 column (Sephadex G-25; Pharmacia) to remove host cells and serum proteins by a modification of the method of Taylor et al. (21). Adult male and female worms were obtained from the subcutaneous tissues of jirds with patent infections.

***In vitro* culture.** *A. viteae* L3 larvae were collected with a glass capillary, counted, washed once with RPMI 1640 medium, and centrifuged at 600 × g for 5 min. The larvae were resuspended in parasite culture medium (PCM) (RPMI 1640 medium supplemented with 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.3], 4 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml [Gibco]).

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FIG. 1. Western blot analysis of purified B20-MBP (lanes 2, 4, 6, and 8) and MBP alone (lanes 1, 3, 5, and 7). Equal volumes of proteins were run on an SDS-15% polyacrylamide gel and blotted onto a nitrocellulose filter. Filter strips were probed with sera from preimmune cattle (lanes 1 and 2) or sera from cattle immunized with either normal L3 larvae (lanes 3 and 4), microfilariae (lanes 5 and 6), or irradiated L3 larvae (lanes 7 and 8) of *O. lienalis*. Molecular mass markers are indicated on the right.

Freshly prepared L3 larvae were cultured in batches of 250 to 500/ml of PCM in 24-well microtiter plates at 27 or 37°C in 5% CO₂ in air for 24 h. Microfilariae were cultured under the same conditions in batches of 16,000/ml. At the end of the incubation period, the parasite cultures were centrifuged at 16,000 × g for 10 min and the pellets were snap frozen. Excretory-secretory (ES) products in the supernatant medium were concentrated by acetone precipitation as described by Lucius et al. (15).

Cattle sera. Jersey bull calves from southeastern England were weaned and maintained under conditions free of exposure to *Onchocerca* or other parasitic nematode infections, as described previously (2, 11). They were then infected by subcutaneous inoculation with 600 normal infective larvae of *O. lienalis* or were vaccinated with an equivalent number of organisms that had been radiation attenuated with either 20 or 40 kilorads of ⁶⁰Co γ-radiation. Serum samples were obtained 6 and 12 weeks later from groups of 20 infected or vaccinated animals; the vaccinees were equally divided into groups that received larvae given each dose of ionizing radiation. In a separate experiment, cattle were immunized against the microfilariae of *O. lienalis* with sonicated *O. lienalis* microfilariae. Sera were collected from vaccinated animals postmortem, 10 days after inoculation of the challenge organism (24).

Expression of recombinant protein. The original cDNA isolated by differential immunoscreening, designated B20, contains a 311-bp insert encoding a single open reading frame of 77 amino acids (1). The insert from the cDNA clone was excised by digestion with *Eco*RI and subcloned into the pMALcR1 expression system of New England Biolabs (Beverly, Mass.). Expression and purification of the B20-maltose-binding protein (MBP) fusion protein were carried out according to the protocol provided by the manufacturer. To obtain nonfused B20, B20-MBP was digested overnight at room temperature with factor Xa enzyme at a concentration of 1 mg/50 ml of 20 mM Tris (pH 8.0)–100 mM NaCl–2 mM CaCl₂ containing 50 mg of fused protein. The cleaved product (~10 kDa) was separated from MBP (43 kDa) after digestion by collecting the filtrate following passage through Ultrafree-Mc 30,000-nominal-molecular-weight-limit filters (Millipore).

Antibody production and affinity purification. Antibodies were raised against B20 in rabbits by subcutaneous injection with 200 μg of B20-MBP mixed with Ribi adjuvant (Universal Biologicals Limited). Booster immunizations were given on three occasions, using 200 μg of cleaved B20 in adjuvant. The rabbits were bled 10 days after the final booster injection. Antibodies were affinity purified as described by Lyon and Weber (16) by plating out the B20 clone (in λgt11) and adsorbing the β-galactosidase fusion protein onto isopropyl-β-D-thiogalactopyranoside (IPTG)-soaked nitrocellulose filters. The filters were incubated overnight in blocking solution consisting of phosphate-buffered saline (PBS)-bovine serum albumin (BSA) (3%, wt/vol) and then incubated with the rabbit antiserum, which had been preadsorbed against *Escherichia coli* lysate. Affinity-selected antibodies were then eluted from the filters with 200 mM glycine buffer (pH 2.6) and neutralized immediately with 1 M Tris-HCl, pH 8.0 (1/10 volume), containing BSA (1%) as a carrier.

Western blotting (immunoblotting). Parasite extracts were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (2% SDS–5% β-mercaptoethanol in 62 mM Tris-HCl, pH 6.8) containing a cocktail of the following protease inhibitors: 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], 0.2 mM TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone), 1 mM N-ethylmaleimide, 0.1 mM TPCK (N-tosyl-L-phenylalanine chloromethyl ketone), and 2 mM phenylmethylsulfonyl fluoride (all from Sigma). Samples were boiled for 5 min and centrifuged at 16,000 × g for 10 min, and the supernatants were fractionated by

FIG. 2. Identification of native OvB20 in different developmental stages of *O. volvulus*. Lane 1, adult male; lane 2, adult female; lane 3, L3 larvae; lane 4, microfilariae. Molecular mass markers are indicated on the right.

SDS-15% (wt/vol) PAGE. Low-molecular-mass markers (14 kDa, α-lactalbumin; 20 kDa, soybean trypsin inhibitor; 30 kDa, carbonic anhydrase; 43 kDa, ovalbumin; 67 kDa, BSA; and 94 kDa, phosphorylase b) (Pharmacia) were run alongside parasite material. Proteins were transferred to nitrocellulose filters by semidry electroblotting with a Novablot apparatus (LKB/Pharmacia). Immunodetection was performed essentially as described by Towbin et al. (23). Bound antibodies were visualized with peroxidase-conjugated goat anti-rabbit immunoglobulin G and 4-chloro-1-naphthol (Boehringer Mannheim Biochemicals).

Immunoelectron microscopy. A solution containing 2% paraformaldehyde and 0.5% glutaraldehyde in 0.075 M sodium cacodylate was cooled to 4°C. Specimens were dissected into pieces no larger than 1 mm³, fixed for 1 h at 4°C, and washed overnight in 0.075 M sodium cacodylate–0.2 M sucrose at 4°C. The tissue was dehydrated at room temperature by using a graded series of ethanol up to 95% before infiltration overnight with LR White resin (Agar Scientific). The tissue was embedded in fresh resin in gelatin capsules and polymerized by incubation at 55°C for 24 h. Ultrathin 90-nm-thick sections were prepared and mounted on Formvar-coated nickel grids.

An immunoglobulin sandwich technique was used to localize the native OvB20 (17). Sections were blocked by incubation in 1% BSA in PBS–0.01% Tween 20 (BPT) for 30 min at room temperature. Affinity-purified rabbit antibodies were incubated with the sections for 2 h. Grids were washed in BPT and exposed to protein A-gold conjugate (Sigma) (10-nm-diameter gold colloid) in the same diluent for 30 min at room temperature. They were then washed sequentially in BPT, PBS-Tween, and distilled water and counterstained in a saturated solution of aqueous uranyl acetate. Sections were examined with a Philips 301 transmission electron microscope.

Vaccinations. (i) *O. lienalis* microfilariae in mice. To investigate the mechanisms of immunity to *Onchocerca* microfilariae in vivo, a mouse model that mimics invasion and establishment of microfilariae in the skin has been developed (25). It utilizes microfilariae of *O. lienalis*, which in mice reciprocally cross-protect against *O. volvulus* microfilariae (5). BALB/c mice (male, 6 to 8 weeks old) were immunized by subcutaneous injection in the lumbar region with three doses of 30 μg of recombinant fused protein (B20-MBP) with complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and PBS on successive occasions at 2-weekly intervals. Control groups were immunized with the MBP and adjuvant alone. Two weeks after the last inoculation, the animals were infected with 5,000 *O. lienalis* microfilariae by subcutaneous injection at the nape of the neck. The establishment of the parasite in the ears was determined 2 weeks after inoculation as described by Townson and Bianco (25).

(ii) *A. viteae* in jirds. To determine whether B20-MBP could induce protection in a fully permissive filarial host-parasite system, we used the *A. viteae*-jird model (28). In an immunization protocol similar to that described for mice, male jirds, 8 to 10 weeks old, were immunized by subcutaneous injection in the lumbar region with three doses of 30 μg of B20-MBP with CFA, IFA, and PBS on successive occasions at 2-weekly intervals. A control group received equivalent quantities of the MBP and adjuvant. Two weeks after the last inoculation, the jirds were infected with 100 *A. viteae* L3 larvae by subcutaneous injection at the nape of the neck. Sixteen weeks after infection, the adult worm burden was assessed as described previously (28), and the sex ratio and lengths of the worms were determined. In a repeat experiment, prior to necropsy, levels of microfilariaemia were determined from a 10-μl sample of heparinized tail blood fixed in 1% formal saline. The following day, adult worm burdens were determined.

Statistical analysis. Data from vaccination experiments were analyzed for statistical significance by using the Mann-Whitney U test. A *P* of <0.05 was taken to be of biological significance.

FIG. 3. Ultrastructural localization by immunoelectron microscopy of the native OvB20 protein. Thin sections of *O. lienalis* L3 larvae were incubated first with affinity-purified anti-B20 antibodies and then with protein A coupled to 10-nm-diameter gold particles for indirect antigen localization ($\times 62,000$). H, hypodermis; M, muscle block; C, cuticle. Bar = 50 nm.

RESULTS

Specific-antibody recognition of B20 following irradiated-larva vaccination. Clone B20 encodes 77 amino acids at the C terminus of the longest clone originally selected by immunodifferential screening of an *O. volvulus* cDNA library using

antibodies from cattle vaccinated with irradiated L3 larvae of *O. lienalis* (1). To characterize the protein encoded by this clone, the insert was subcloned in the vector pMALcR1, which expresses the B20 recombinant protein fused to the MBP. The fused protein was affinity purified on an amylose resin column

and analyzed by Western blotting. Figure 1 shows that the purified fused protein (B20-MBP) strongly reacted with sera from cattle immunized with irradiated L3 larvae of *O. lienalis* but did not react with sera from preimmune cattle, cattle infected with normal L3 larvae of *O. lienalis*, or cattle immunized with microfilariae of *O. lienalis* (24).

Stage-specific distribution of native OvB20. Fused protein was cleaved with factor Xa and used to raise antibodies as described in Materials and Methods. Affinity-purified anti-B20 antibodies were used to probe Western blots containing protein extracts from the different developmental stages of the *O. volvulus* life cycle. Figure 2 shows that the antibodies reacted with a single band of ~42 kDa in microfilariae and two bands of ~52 and 65 kDa in the L3 stage. No reaction was detected in the adult stages. The 65-kDa band in the L3 stage was found to be lost if the larvae were washed extensively in PBS before solubilization in sample buffer (data not shown).

Ultrastructural localization of OvB20. As the original cDNA was isolated with antibodies raised against irradiated *O. lienalis* larvae, this species was used to characterize the ultrastructural localization of the native protein. Immunoelectron microscopy revealed that the affinity-purified anti-B20 antibodies reacted with components found in discrete patches in the hypodermis and overlying cuticle of L3 larvae. Within the cuticle, labelling was observed in the basal and median zones (Fig. 3). This pattern was not visualized in sections of microfilariae or adults (data not shown). No labelling was evident in sections of L3 larvae or adults when preimmune rabbit serum was tested.

Characterization of a homologous native protein in *A. viteae*. We have demonstrated elsewhere strong hybridization of B20 to *A. viteae* genomic DNA (1). Because of the scarcity of *O. volvulus* material, we decided to use the rodent parasite as a model to characterize and study the dynamics of the expression of the protein in more detail. Western blot analysis using protein extracts from the different developmental stages of the *A. viteae* life cycle and the same affinity-purified anti-B20 antibodies confirmed the larval specificity of OvB20. As for *O. volvulus*, a band of 65 kDa was detected in *A. viteae* L3 larvae (Fig. 4, lane 3). The lower-molecular-mass band observed in *O. volvulus* microfilariae (Fig. 2), however, was generally not observed in *A. viteae* microfilariae. No reaction with extracts of adult worms was detected (Fig. 4, lanes 1 and 2). When L3 larvae of *A. viteae* were cultured in serum-free medium for 24 h at 37°C, the 65-kDa band could be detected in the larval ES products (Fig. 4, lane 5). The secretion of the 65-kDa product appeared to be reduced when the L3 larvae were cultured at 27°C, although this may simply reflect an overall reduction in protein secretion observed in larvae cultured at 27°C (Fig. 4, lane 4). No product was detected in the ES products of cultured microfilariae (data not shown).

Vaccination with B20-MBP. Vaccination of mice with B20-MBP did not induce a reduction in numbers of microfilariae recovered from the ears 2 weeks after inoculation. The mean ($\pm$ standard deviation) recoveries were 159 ($\pm$ 48) for vaccinated animals and 159 ($\pm$ 69) for the controls.

In contrast, immunization against *A. viteae* of jirds resulted in significant reductions in numbers of adult worms 16 weeks after infection (Fig. 5A, 60% reduction in experiment 1; Fig. 5B, 49% reduction in experiment 2; $P < 0.05$ in both experiments). Male and female worms were equally affected in each of the experiments. There was no significant stunting of worms recovered from vaccinated animals compared with those from control groups (data not shown). A major reduction (97%) in the levels of microfilariae from vaccinated jirds was observed in experiment 2 (Fig. 5C). This may be attributed to a direct effect

FIG. 4. Detection of the OvB20-cross-reactive native proteins of *A. viteae* in somatic extracts (lane 1, adult male; lane 2, adult female; lane 3, L3 larvae) and ES products (lanes 4 and 5, ES products from L3 larvae cultured at 27 and 37°C, respectively) by Western blotting with anti-B20 antibodies. Samples were prepared as described in Materials and Methods and run on an SDS-15% polyacrylamide gel. Molecular mass markers are indicated on the right.

on the microfilariae or to events affecting the adults, such as reduced worm burdens or antifecundity effects.

DISCUSSION

We have reported elsewhere the isolation of a cDNA of *O. volvulus* selected by preferential recognition of antibodies from irradiated-larva vaccination with the related cattle parasite *O. lienalis* (1). The original 311-bp clone, designated B20, was used to isolate a 1,478-bp cDNA, OvB20. This clone codes for 466 amino acid residues, hybridizes with a 1.6-kbp transcript, and appears to be transcribed from a filaria-specific, single-copy gene. Experiments incorporating in vitro transcription and translation of the OvB20 cDNA suggest that the product of the gene undergoes co- or posttranslational modification. A polypeptide of 53 kDa, consistent with the predicted amino acid sequence, and a product of 42 kDa were detected. In the presence of canine microsomes, a glycosylated product of 65 kDa was formed (1). These features of the OvB20 clone appear to be consistent with the different forms of native OvB20 products described in this report. The 65-kDa protein expressed in L3 larvae and detected in culture supernatants is probably a modified version of the 52-kDa protein containing carbohydrate and/or lipid side chains, whereas the 42-kDa product detected in *O. volvulus* microfilariae may be the result of proteolytic cleavage.

OvB20 appears to be a protein with a developmentally regulated expression confined principally to larval stages. The intriguing localization of the native protein to discrete patches of the hypodermis and cuticle of infective larvae together with the detection of the protein in culture supernatants suggests that OvB20 is a secreted product originating from the hypodermis. Indeed, extensive washing of intact infective larvae prior to electrophoretic analysis can lead to the loss of the protein (unpublished observation). The lack of detectable protein in adult worms implies that the cDNA derived from an adult worm library originated from embryos or microfilariae

FIG. 5. Recoveries of *A. viteae* adult worms and microfilariae from groups of vaccinated and control jirds 16 weeks after inoculation with challenge organisms. (A) Experiment 1, adult worm recovery. (B) Experiment 2, adult worm recovery. (C) Experiment 2, peripheral blood microfilaremia. Bars represent mean values for each experimental group. In experiment 1, $n = 7$ for each group; in experiment 2, $n = 6$ for the challenge control group and $n = 4$ for the vaccinated group.

developing in utero and is present in uterine stages in low abundance, below the levels of detection used in this study.

Characterization of a homologous protein in *A. viteae* with the same monospecific antibodies confirmed the distinct stage-specific expression of the 65-kDa protein in L3 larvae. Detection of the lower-molecular-mass product in microfilariae was inconsistent and rarely observed, suggesting that a further level of regulation may occur within individual developmental stages. These observations are consistent with in situ hybridization experiments with different developmental stages of *O. volvulus* which indicate that transcription is initiated in embryos and is maximal during L2 to L4 development (1).

We subcloned B20 into the pMALcR1 expression system in order to test the potential of the cloned cDNA as a vaccine candidate. We wished to validate that the new construct retained the selection criteria of the original cDNA. Immunoblotting analysis confirmed the specific correlation between the produced recombinant antigen and vaccination with irradiation-attenuated *O. lienalis* infective larvae in cattle, which was the basis of the original selection of this clone (1).

Immunization of jirds with B20-MBP induced a 49 to 60% reduction in adult worm recoveries, with a 97% reduction in circulating microfilariae. Male and female worms were equally affected by vaccination, and the surviving parasites showed no evidence of developmental or growth retardation. The marked reduction in peripheral microfilaremia in vaccinated animals probably reflects the reduction in adult worm numbers or fitness. The restricted developmental expression of the protein indicates that larval stages (L3 and L4) are the probable target of immune attack. The lack of protective efficacy against *O. lienalis* microfilariae may relate to the differential expression of OvB20 between stages and suggests that it is the expression of the high-molecular-weight secreted forms which confers susceptibility to immune prophylaxis. Native secreted antigens of infective larvae have previously been shown to be potent immunogens against *A. viteae* infection (15).

Induction of partial immunity to *A. viteae* by immunization with B20 suggests that heterologous protection by antigens conserved among filarial worms can occur. It is possible that vaccination against *Onchocerca* infective larvae or inoculation

with the homologous antigen of *A. viteae* may improve the efficacy of B20-induced immunity. The conservation of genes similar to OvB20 in other filarial genomes (1) suggests that homologs from other filarial species, such as *Brugia malayi* and *Wuchereria bancrofti*, may be suitable candidates for evaluation as vaccine components for human lymphatic filariasis.

The successful use of radiation-attenuated larvae in inducing protective immunity in animal models of filariasis is based on the proven efficacy of live attenuated vaccines for veterinary nematode parasites (10). However, none of these vaccines are antigenically defined. It is possible that OvB20 is up-regulated in irradiated larvae as a component of a stress response by the parasite. Preliminary studies with cultured irradiated *A. viteae* larvae lend support to this hypothesis (unpublished observation). Alternatively, the attenuation of parasite development induced by irradiation may result in a prolonged exposure to antigens with stage-specific expression, such as OvB20. Although OvB20 appeared to be exclusive in its preferential recognition by *O. lienalis* irradiated vaccination sera (1), a similar approach of preferential antigen recognition by antibodies from jirds immunized with irradiated *B. malayi* infective larvae has identified filarial paramyosin as a candidate protective antigen (13). Vaccination with recombinant filarial paramyosin has also been shown to induce partial immunity to *B. malayi* infection in jirds (14).

Although the development of vaccines against human filarial parasites is still at an early stage, the identification of antigens with protective efficacy is encouraging and provides the opportunity to characterize the epitopes and mechanisms of immunity suitable for the construction of subunit vaccines similar to those recently showing promise against malaria (20). We are investigating a variety of delivery mechanisms and molecular manipulations designed to improve the efficacy of B20, prior to vaccination trials in bovine models of onchocerciasis.

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