An Immunogenic *Onchocerca volvulus* Antigen: A Specific and Early Marker of Infection

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Onchocerciasis (river blindness) is a serious health problem and a severe obstacle to social and economic development, especially in Africa. A complementary DNA fragment coding for an *Onchocerca volvulus* antigen (OV-16) was cloned and expressed in the plasmid vector pCG808fx. Immune responses to this *O. volvulus*–specific recombinant antigen were detectable in patients with documented onchocerciasis; the antibody response was also detectable at 3 months and at more than 1 year before infection could otherwise be detected in humans and in chimpanzees experimentally infected with *O. volvulus* third-stage larvae.

THE PARASITIC FILARIAL NEMATODE *Onchocerca volvulus* is the causative agent of onchocerciasis. It infects approximately 18 million people in Africa and Latin America, manifesting itself as a severe disease of the skin and eyes ("river blindness") (*1*). Although vector control and the advent of ivermectin promise a drastic reduction of the disease, there is a crucial need for accurate diagnosis of early and light infections in areas where vector control has been established or where chemotherapeutic control is envisaged (*2*). Definitive diagnosis currently relies either on detecting microfilariae (mf) in the skin or eyes or in identifying the adult worm in subcutaneous nodules that are surgically removed, techniques that are invasive and relatively insensitive. Hitherto, serological tests have not been satisfactory, partly because of the broad antigenic cross-reactivity among the filarial and other helminth parasites of humans and partly because they fail to detect early and low-level infections (*1*). A species-specific diagnosis also has prime importance because treatment differs for different filarial infections.

Low molecular weight (LMW) antigens of *O. volvulus* have greater species specificity than antigens of higher molecular weight (*3*). Using affinity-purified antibodies against such LMW parasite antigens, we have identified and characterized OV-16, a cDNA clone whose specificity was analyzed by the immunoblotting technique (*4*). It has an open reading frame that encodes a 152–amino acid polypeptide containing a 16–amino acid putative signal peptide; the OV-16 molecule could therefore be excretory or secretory in nature (*4*). In the present study, we describe the overexpression and isolation of the OV-16 antigen and show that, because it is highly immunogenic early in infection and also is *O. volvulus*–specific, it can be exploited to identify the presence of *O. volvulus* parasites even in the early prepatent period of infection when mf in the skin or adults in nodules cannot be detected.

A 682-bp fragment of the OV-16 gene that lacked the leader sequence (123 amino acids) was fused to the COOH-terminus of the maltose-binding protein (MBP) encoded by *malE* of *Escherichia coli* (*4, 5*). The construction of the recombinant plasmid pCG808fx-16 has been described in (*6*). The signal peptide was not included in the construct as hydrophobic regions of eukaryotic proteins are frequently toxic to *E. coli* (*7, 8*). The purification of the fusion protein MBP-16 (*9*) is illustrated in Fig. 1A. First, there was a major protein band with a relative molecular mass (M_r) of 52,000, which became prominent after induction with 0.3 mM isopropylthiogalactoside (IPTG) (lane 2). Next, the MBP-16 fusion protein was purified by affinity chromatography on cross-linked amylose (*10*). The major band at M_r 52,000 continued to be present, along with a minor band of M_r 40,000, which probably represented a premature termination of the fusion protein or its digestion by *E. coli* proteases (lane 4). Between the MBP and the OV-16 domains (*11, 12*) this fusion protein contains the recognition sequence Ile-Glu-Gly-Arg for the activated blood clotting factor X_a, a protease. Digestion of MBP-16 with factor X_a allowed separation of the two protein domains (Fig. 1A, lane 5). Some of the fusion protein remained uncleaved. We fur-

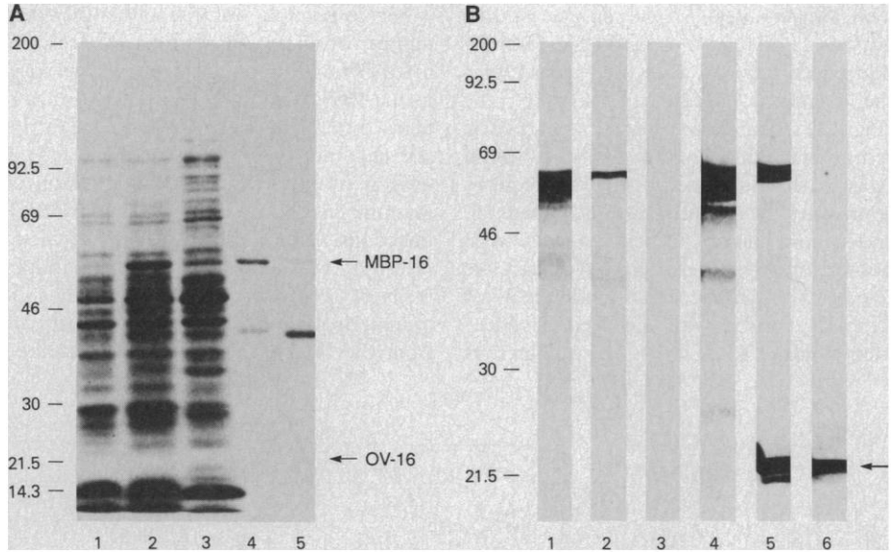

Fig. 1. (**A**) SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the expression of the MBP-16 fusion protein and of the subsequent separation of the protein domains. In the SDS-PAGE (5 to 15% gradient gels) we used 40 μg of *E. coli* lysate without IPTG induction (lane 1), 40 μg of cell lysate after induction (lane 2), 40 μg of flow-through from the amylose column (lane 3), 5 μg of purified MBP-16 eluted from the cross-linked amylose column (lane 4), and 5 μg of MBP-16 after digestion with factor X_a for 4 days at room temperature (lane 5). Molecular size standards were from Amersham. (**B**) Protein immunoblot analysis of the isolated *O. volvulus* antigen (OV-16). Identical gels (4 to 20% gradient) with 5 μg of MBP-16 (lanes 1 and 4), 5 μg of factor X_a–digested MBP-16 (lanes 2 and 5), and 200 ng of the FPLC-isolated OV-16 (lanes 3 and 6) were blotted onto nitrocellulose. They were probed with rabbit antiserum raised against MBP (1:20,000) (lanes 1, 2, and 3) or with a pool of sera from patients with onchocerciasis [lanes 4, 5, and 6 (1:500)]. Bound antibodies were visualized by a second incubation with alkaline phosphatase–conjugated goat antibody to rabbit or human immunoglobulin G (IgG).

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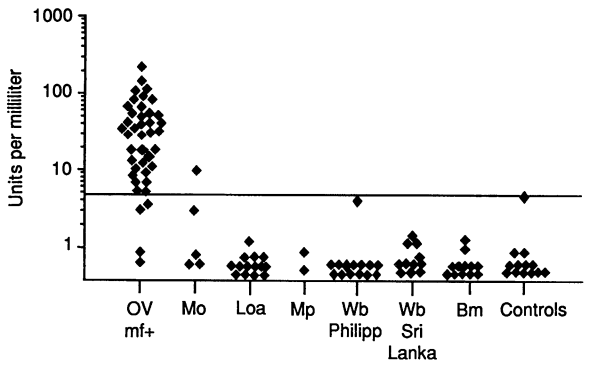

Fig. 2. Detection of *O. volvulus*–specific antibodies (total IgG) in onchocerciasis patients OV mf+ (n = 41) by use of the recombinant OV-16 antigen in an ELISA (*14*). Sera were also used from clinically well-defined filarial infections including *M. ozzardi* (Mo) (n = 5), *M. perstans* (Mp) (n = 2), and *L. loa* (Loa) (n = 14), as well as sera from patients with lymphatic filariasis caused by *W. bancrofti* (Wb) (n = 25) from the Philippines (Philipp) or Sri Lanka or by *B. malayi* (Bm) (n = 12). The solid line represents the cut-off value calculated as the mean of the control sera (unexposed individuals living in Switzerland; n = 13) plus three standard deviations. Values less than 1.9 units per milliliter are considered to be at background level.

ther purified OV-16 from the MBP, the truncated form, and the uncut fusion protein by fast protein liquid chromatography (FPLC) with the use of a Mono S column. The purification procedure did not affect antigenicity as determined by protein immunoblot analysis of the fusion protein and purified OV-16 (Fig. 1B).

The purified OV-16 was used in an enzyme-linked immunosorbent assay (ELISA) to analyze the antibody responses of 41 onchocerciasis patients (ages 3 to 60 years) (*13*), who had proven infections (mf detected in skin) and were residents of a region highly endemic for *O. volvulus* in the savanna zone of Mali. Sera from patients with other filarial infections—*Wuchereria bancrofti, Brugia malayi, Mansonella ozzardi, Loa loa, Mansonella perstans*—and other helminthic infections (*13*) were used to ascertain the diagnostic specificity of OV-16 (*14*) (Fig. 2). In our hands, with this particular recombinant antigen, an ELISA is more sensitive than immunoblot analysis, requires approximately one-fifth as much material, and is more practical to perform when large populations are to be studied. We were able to detect OV-16–specific antibodies in 37 of 41 (90%) patients with onchocerciasis (geometric mean 41.4 units per milliliter as compared to normal levels of <4.6 units per milliliter) and were also able to differentiate onchocerciasis from the other filarial infections, including *L. loa* (0/14 positive), *W. bancrofti* from the Philippines (0/14 positive) and Sri Lanka (0/11), *B. malayi* from Indonesia (0/12), and *M. perstans* from West Africa (0/2). For *M. ozzardi*, the one individual (of five studied) from Venezuela who reacted positively in the ELISA resided in an area where coinfection with *O. volvulus* was a distinct possibility. Unexposed persons living outside filarial endemic areas (Switzerland) had no antibody to this protein (0/13 positive). Thus, by using this assay, we obtained a specificity of 98% (1/57) and a sensitivity of 90% (37/41) for *O. volvulus*.

No correlation was found between the number of mf per skin snip and the reactivity to OV-16, although there was a decrease in the levels of antibody to OV-16 in patients over 20 years of age with mf in the skin as compared to younger patients (*15*), a finding consistent with the modulation of immune responses seen in chronic parasitic infections. Although the species specificity of OV-16 was dramatic (Fig. 2), the antigenicity of OV-16 was conserved among geographic isolates of *O. volvulus* in patients from the West African savanna (described here), as well as those from the West African rain forest (Ivory Coast) and from the New World (Guatemala) (*16*).

Polyclonal antiserum raised to purified OV-16 demonstrated that OV-16 accumulated in parasite-free culture supernatants in its posttranslationally modified forms (*17*), with M_rs in the range 22,000 to 30,000. This finding suggested that OV-16 is a released parasite product and would be available to induce an immune response early in infection. To examine this possibility more directly, we monitored the course of the humoral immune response to OV-16 in parallel with the onset of patency (first detection of mf in the skin) in two chimpanzees experimentally inoculated with infective OV larvae (*18*). In these chimpanzees, antibodies to OV-16 developed 3 months and 12 months before the first appearance of skin mf (Fig. 3, A and B).

Because infection of children can be an important epidemiologic indicator of ongoing transmission of *O. volvulus*, we analyzed the antibody response to OV-16 in eight exposed but parasitologically negative children (ages 1 to 14), who were part of a 4-year longitudinal study (*13*) carried out in a savanna region of Mali highly endemic for onchocerciasis (Table 1). In three of the children, *O. volvulus* infection could be detected by the presence of antibodies to OV-16 during the prepatent period, 1 to 1.5 years before the appearance of mf in the skin. In four other children, significant levels of antibody were observed in the same year that mf appeared in the skin. In the eighth child, no clinical, parasitological, or serological evidence of infection was observed, and, therefore, he was assumed to be truly uninfected.

The use of recombinant antigen OV-16 overcomes many of the problems that previously plagued the diagnosis of onchocerciasis: lack of parasite material, poor specificity and sensitivity of assay, and

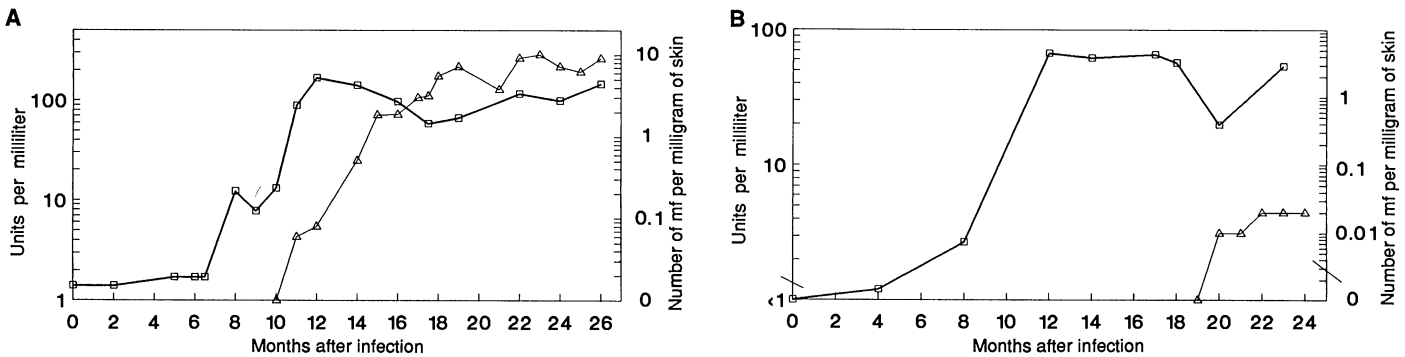

Fig. 3. Seroreactivity against OV-16 in experimental *O. volvulus* infection in chimpanzees. Each animal was inoculated with 250 ± 5 infective third-stage (L_3) larvae of *O. volvulus*. Detailed parasitological examinations were carried out monthly from 3 months before inoculation until 38 months after inoculation (*18*). An ELISA was carried out as described (*14*). Chimpanzees showed a positive titer against OV-16 at 3 months (**A**) and at 12 months (**B**) before the time that mf were first detected in skin snips; □, ELISA; △, number of mf per milligram of skin.

Table 1. Detection of antibodies to OV-16 in a longitudinal study of children (ages 1 to 14) from a savanna region in Mali highly endemic for onchocerciasis. ND, not done.

Child	Anti–OV-16 Ab* in year				Skin mf present in year			
	1	2	3	4	1	2	3	4
1	−	+	+	+	−	−	+	+
2	−	+	+	+	−	−	+	+
3	+	+	+	+	−	−	+	+
4	−	+	+	ND	−	+	+	ND
5	−	+	+	ND	−	+	+	ND
6	−	−	+	+	−	−	+	+
7	−	+	+	+	−	+	+	+
8	−	−	−	−	−	−	−	−

*A positive value (+) is defined as >3 SD above the geometric mean of 13 normal individuals run simultaneously.

insensitivity in detecting prepatent and low-level infections. Over most of the large area of the Onchocerciasis Control Program in West Africa, *O. volvulus* transmission has been interrupted by vector control (*1, 2, 19*). However, reinvasion of infective black flies occurs in some border areas and is responsible both for recurrent infections (*1, 20*) and for infections of previously unexposed children (born after the establishment of effective vector control). The use of OV-16, or of similar antigens, should allow the early and specific diagnosis of new infections or reinfections in such vector reinvasion areas, as well as the detection of light infections in areas where control is being attempted by widespread use of ivermectin (*21*). Such a capability will be of paramount importance in monitoring, evaluating, and consolidating onchocerciasis control by both vector control and chemotherapeutic strategies.

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6. We obtained the recombinant plasmid by ligating a 682-bp fragment of OV-16 (*4*) into the Eco RI site of pCG808fx (*5*). This plasmid contains a portion of the *malE* gene with its signal sequence fused to the *lacZ* coding sequence. The ligation mixture was used to transform *E. coli* 71-18. We identified transformants expressing the *O. volvulus* OV-16 antigen by protein immunoblot, using a pool of sera from patients with onchocerciasis.
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9. *Escherichia coli* 71-18Δ [(*lac-proAB*) *thi supE* (F′ *proA*$^+$*B*$^+$ *lacI*^{q} ZΔM15)] bearing the appropriate plasmid construct was grown at 37°C in 250 ml of Luria broth to 0.8 O.D. units at 600 nm and induced with IPTG for 2 hours. The cells were harvested by centrifugation at 9 × 10^3 rpm for 15 min, 4°C. The cell pellet was washed twice in cold phosphate-buffered saline, pH 7.5, and 5 mM EGTA and suspended in 25 ml of lysis buffer [10 mM tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol, *N*-tosyl-L-phenylalanine-chloromethyl-ketone (20 μg/ml), leupeptin (10 μg/ml), 20% sucrose, 30 mM NaCl, 10 mM EDTA, and 0.2% Tween 20]. Cells were lysed by sonication, and unbroken cells and cell debris were removed by centrifugation for 30 min, 4°C, at 10 × 10^3 rpm. The supernatant was diluted 1:5 with a solution of 10 mM tris-HCl, pH 7.5, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 1 mM PMSF, and 10 mM EGTA and adsorbed overnight at 4°C with 25 ml of cross-linked amylose resin. The bound fusion protein was eluted with maltose, and fractions were collected, pooled, and dialyzed to remove maltose. The dialyzate was concentrated and the protein content estimated (*5, 10*). Approximately 6 mg of fusion protein were obtained from 250 ml of culture supernatant. The digestion of MBP-16 fusion protein (1 mg) with 10 μg of factor X_a for 4 days at room temperature resulted in the cleavage of approximately 60 to 70% of the fusion protein. The cleavage products were separated by FPLC with the use of a Mono S column.
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14. Levels of anti–OV-16 were determined by ELISA. Briefly, Immulon 4 plates (Dynatech Laboratories) were coated with 300 ng of OV-16 per milliliter of coating buffer, pH 7.6, and incubated overnight at 4°C. The plates were blocked with 5% bovine serum albumin for 1 hour at 37°C. All sera were run in duplicate at a 1:100 dilution and incubated overnight. For assays of total IgG, Fc-specific, alkaline phosphatase–conjugated goat antibody to human IgG (Sigma) was added. Thirteen uninfected samples were used to determine the normal range (mean ± 3 SD). A high-titer standard reference onchocerciasis serum pool was used to generate calibration curves against which all sera were compared for antibody levels (Flow Cytometric Programme 1.5, Munich, Germany). Levels are expressed as arbitrary units per milliliter. There was no recognition of OV-16 by antibodies of the IgE isotype.
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A Multisubunit Ribozyme That Is a Catalyst of and Template for Complementary Strand RNA Synthesis

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Derivatives of the *sunY* self-splicing intron efficiently catalyzed the synthesis of complementary strand RNA by template-directed assembly of oligonucleotides. These ribozymes were separated into three short RNA fragments that formed active catalytic complexes. One of the multisubunit *sunY* derivatives catalyzed the synthesis of a strand of RNA complementary to one of its own subunits. These results suggest that prebiotically synthesized oligonucleotides might have been able to assemble into a complex capable of self-replication.

RNA MOLECULES CAPABLE OF SELF-replication are postulated to have been important in the early evolution of life (*1–4*). However, an RNA replicase probably requires a folded structure to carry out efficient catalysis, while an optimal template is unstructured. These conflicting requirements may be accommodated by a multisubunit RNA replicase, with the dissociated components serving as templates and the assembled complex as an RNA polymerase. The group I self-splicing introns catalyze phosphodiester exchange reactions similar to those catalyzed by DNA and RNA polymerases (*1*). On the basis of this natural catalytic capacity, these introns might be modifiable to function as RNA polymerases. Indeed, the intron from *Tetrahymena* catalyzes complementary strand RNA (cRNA) synthesis on external templates (*5*). However, this reaction is quite inefficient. Less than 1% of a 40-nucleotide (nt) template is copied to a full-length complementary strand. Self-replication of a ribozyme as large as the *Tetrahymena* intron (413 nt) is clearly impossible with such an inefficient reaction. Smaller ribozymes that are more efficient at cRNA synthesis are required if self-replication is to be demonstrated. We have addressed this question by studying the properties of modified forms of the self-splicing intron *sunY* from bacteriophage T4.

The conserved core of the *sunY* intron

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