Prevalence of Antibodies to *Onchocerca volvulus* in Residents of Oaxaca, Mexico, Treated for 10 Years with Ivermectin

Alberto Gómez-Priego,1,2 Raymundo Mendoza,1 and Jorge-Luis de-la-Rosa1*

Laboratorio de Helmintos Tisulares, Instituto de Diagnóstico y Referencia Epidemiológicos, Secretaría de Salud, Santo Tomás, Miguel Hidalgo,1 and Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, Coyoacán,2 México

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Onchocercosis is a filarial infection that causes ocular and skin disease in human beings; it has been considered one of the main causes of blindness in the world. Areas of endemicity are located in the western and central zones of Africa and in six Latin American countries. A number of planned actions have been carried out in Africa and in America in order to control the disease. Since 1991, in all of the Latin American foci of endemicity and particularly in Mexico, control actions have been primarily based on ivermectin administration twice a year. Thus, it is expected that worm transmission will be interrupted at the end of this decade (2, 17, 21). To be certain that transmission is interrupted, the ELISA procedures were first evaluated. Serological studies were performed with serum samples from skin microfilaria carriers from Guatemala and from people microfilarioi demic negative living in the same area as the Guatemalan patients. Vectors collected in the field; (ii) early ocular injuries (i.e., keratitis punctata) are not detected in residents of areas of controlled endemicity; and (iii) anti-*Onchocerca volvulus* antibodies are absent in children under 16 years old, or, alternatively, seronegative conversion occurs in people previously identified as seropositive to *O. volvulus* antigens (6).

Humoral immune response is a constant finding in onchocercosis infection, but it has been documented that antibody prevalence at community level is not uniform, since rates depend on endemicity level and intensity of infection in the localities (5, 8, 9). In addition, it has been considered that antibody titers must decline sometime after successful microfilaricide treatment with ivermectin. However, very few studies have been performed to study this aspect. In fact, evidence was found that, in a Mexican area of hyperendemicity, the antibody prevalence is decreased from 24% to 4% after 5 years of biannual ivermectin treatment (18). Although it is known that antibodies fail to distinguish between present and past infections, a good correlation between current infection and immunoglobulin G4 (IgG4) isotype detection has been observed (3, 12, 13, 20). However, the time of antibody persistence in treated onchocercosis patients is actually unknown. Frequent cross-reactivity found with human lymphatic filariosis serum samples has been observed in *Onchocerca* antibody assays (15, 16). Nevertheless, in Mexico cross-reactions are not expected because other filarial infections have not been reported.

Since the serological aspects of onchocercosis have been poorly studied in Mexico, the aim of this study was to evaluate an enzyme-linked immunosorbent assay (ELISA) to detect IgG and IgG4 antibodies, using a crude extract prepared from *O. volvulus* adult worms as antigen. The validated ELISA was then used to determine the filarial antibody status of people who have received ivermectin treatment twice a year for 10 years.

**MATERIALS AND METHODS**

Serum samples. Five groups of serum samples were studied; all of them were obtained from people between 18 and 79 years old. Samples were equally representative of both sexes. Group 1 (onchocercosis confirmed) was created with 21 samples from Guatemalans with onchocercosis documented by eye or skin injuries (including subcutaneous nodules) compatible with the disease. Infection was confirmed by microscopic demonstration of *O. volvulus* microfilariae emerging from two skin-snip biopsies or, in a few cases, by positive Mazzotti reactions. Group 2 (endemic controls) was formed by 36 samples from healthy people without clinical or parasitological evidence of onchocercosis, living in the same

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*Corresponding author. Mailing address: Laboratorio de Helmintos Tisulares, Instituto de Diagnóstico y Referencia Epidemiológicos, Secretaría de Salud, Cárpolo 470, Santo Tomás, Miguel Hidalgo, México D.F. 11340, México. Phone: 52-55-53427550, ext. 224, Fax: 525-55-53413264. E-mail address for J.-L. de-la-Rosa: jldelarosa@salud.gob.mx. E-mail address for A. G. Priego: E-mail: agpriego@prodigy.net.mx.
area of Guatemala as individuals in group 1. Group 3 (other diseases) was created by samples from people living in various areas of Mexico that were infected with different pathogens: trichinellosis \((n = 5)\), visceral larva migrans \((n = 7)\), neurocysticercosis \((n = 2)\), cystic echinococcosis \((n = 5)\), localized cutaneous leishmaniosis \((n = 5)\), Chagas’ disease \((n = 7)\), toxoplasmosis \((n = 7)\), brucellosis \((n = 6)\), and dengue fever \((n = 6)\). These sera were kindly donated by several laboratories of our institute. Group 4 (nondenendemic controls) was created with 42 samples from donors from an area of nonendemicity (Mexico City); several laboratories, each providing a health certificate, kindly donated these samples. Finally, group 5 (putative onchocercosis) was created with 71 samples from residents from four districts at the area of onchocercosis endemicity of Oaxaca, Mexico (Yentexte, San Isidro Reforma, Santiago Yagallo, and San Juan Juquilla), all considered localities of mesoendemicity by the Mexican Onchocercosis Control Program in 1991. Oaxaca residents have received biannual microfilaricide treatment with ivermectin for 10 years. At the beginning of the treatment, they were considered to be onchocercosis-infected patients since they were positive either as skin snips, subcutaneous nodules, or Mazzotti reaction.

Such a condition was not determined when serum samples were taken for the present study. Coded serum samples were submitted for the immunoassays, and results were compared only on completion of all the assays to ensure blindness in the protocol.

**Crude extract.** *O. volvulus* male and female adult worms (OvAw) were obtained by dissecting subcutaneous nodules surgically removed from Mexican patients of the southern onchocercosis focus of Chiapas state. Nodules were preserved at –20°C and incubated in a 2% ambient temperature in 67% glycerol for several years. After exhaustive washing with 0.85% saline solution, nodules were cut into two or three pieces. Dissected fragments of filarial nematodes were collected in 1 ml of 150 mM phosphate-buffered saline (PBS; pH 7.2) containing the following protease inhibitors: tosylsulfonphenylalanylchloromethyl ketone and N-α-tosyl-λ-lysine chloromethyl ketone (at 50 μg/ml) and phenylmethylsulfonyl fluoride at 1 mM final concentration (Sigma, St. Louis, Mo.). The weight of the drained OvAw fragments was obtained, and supernatant was saved. Then the suspension was adjusted to 6 ml of PBS per g (wet weight) of parasite fragments, and the first supernatant was added. The preparation was then homogenized with a Potter glass tissue grinder on an ice bath for 15 min. The suspension was transferred to an Erlenmeyer flask and incubated overnight at 4°C with constant stirring and then centrifuged at 22,000 × g at 4°C for 30 min. Supernatant was collected and stored frozen at −70°C in aliquots until use. The protein concentration was determined by a colorimetric method (Bradford dye reagent) following the procedures described by the manufacturer (Bio-Rad Laboratories, Hercules, Calif.).

**ELISA-IgG.** The OvAw crude extract was diluted to 3 μg/ml in 100 mM carbonate-bicarbonate buffer, pH 9.6. Flat-bottom polystyrene plates (Costar, Cambridge, Mass.) were coated at 100 μl/well with the antigen solution, incubated overnight at 4°C, and then washed three times with PBS containing 0.05% Tween 20 (PBS-T). Wells were blocked during 2 h at 37°C with 1% nonfat dry milk (Svehly; Nestlé, Mexico), dissolved in PBS-T, and washed as described above. Individual serum samples (100 μl) diluted 1:1,000 in PBS-T were added to the wells in duplicate, incubated for 2 h at 37°C, and washed again. Anti-human IgG-horseradish peroxidase conjugate (Zymed, San Francisco, Calif.) diluted 1:4,000 in PBS-T was added, and the mixture was incubated for 2 h at 37°C. After washing, the color reaction was developed with the addition of a substrate reaction mixture solution prepared with o-phenylenediamine (Sigma) and H₂O₂ (Merck, Mexico) dissolved in 75 mM phosphate-citrate buffer, pH 5. The enzymatic reaction was ended with 2 M H₂SO₄ solution after a 15-min reaction, and absorbency values were measured spectrophotometrically with an ELISA reader at 490 nm. Absorbencies higher than 0.363 were considered positive. This number was the mean + 3 times the standard deviation of endemic control sera (group 2).

**ELISA-IgG4.** The ELISA-IgG4 was performed as described for ELISA-IgG, except that a mouse monoclonal anti-human IgG4-biotin conjugate, diluted 1:4,000 in PBS-T (Pharmingen-Becton Dickinson, Franklin Lakes, N.J.), was added to each well. Plates were incubated for 2 h at 37°C and washed as described above, and then 100 μl of avidin-horseradish peroxidase conjugate (Sigma) diluted 1:6,000 with PBS-T was added. Color reaction was developed with the addition of a substrate solution prepared as described above. Absorbency values (490 nm) were obtained with an ELISA reader. The cutoff value (0.357) was calculated as described for ELISA-IgG.

**Determination of serological parameters.** The serological parameters of the ELISAs (e.g., sensitivity, specificity, predictive values, and the kappa coefficient) were determined according to previously described procedures (1).

**RESULTS**

The serological response, measured as \(A_{490}\) obtained by ELISA-IgG and ELISA-IgG4 against the OvAW, was as follows. In ELISA-IgG experiments, the onchocercosis-infected individuals (group 1) had a different response with respect to endemic control and nondenendemic control groups (group 4), as well as with those considered as putatively infected (group 5). The mean absorbency values obtained from group 1 \((0.705)\) were, respectively, 4.9, 4.7, and 4.8 times higher than those obtained from the groups mentioned above, while in ELISA-IgG4, the mean absorbency value of the confirmed onchocercosis group was 0.624. When this value was compared with those of the same groups mentioned above, the resulting quotients were 3.3, 2.6, and 2.7, respectively.

Individual results obtained in all groups are presented in Fig. 1. As can be seen, 15 of 21 \((71\%)\) serum samples from onchocercosis-infected patients were positive by ELISA-IgG. Three out of 36 sera \((8\%)\) of the endemic control group were marginally positive, while the other 3 out of 52 serum samples \((6\%)\) from the group with other diseases were also positive. Interestingly, these three positive samples were from patients infected with the nematode *Trichinella spiralis*. No positive samples were found in the nonendemic control group. Results obtained with ELISA-IgG4 show that 18 of 21 serum samples \((86\%)\) from the *O. volvulus*-infected patients were positive, while no positive samples were obtained from the other groups.

Results of the serological evaluation of ELISA-IgG and ELISA-IgG4 are presented in Table 1. Comparatively, the ELISA-IgG4 is a better test than the ELISA-IgG, as the evaluation rates suggest. For example, sensitivity was 71% for ELISA-IgG and 86% for ELISA-IgG4, while the specificities were 92 and 100%, respectively. Moreover, according to the kappa coefficient index \((0.78)\), detection of total IgG or IgG4 has good concordance.

Only IgG but no IgG4 isotypes were detected in 3 of 70 serum samples from the ivermectin-treated individuals, and these samples show an \(A_{490}\) of 0.835 ± 0.2 on average (Fig. 1). These positive subjects were two males 28 and 34 years old and one female 78 years old, all of them from the locality of Santiago Yagallo, Oaxaca.

**DISCUSSION**

It is expected that onchocercosis will be eliminated from the Latin American foci of endemicity for the next 10 years, provided that high levels of coverage with biannual ivermectin chemotherapy and nodulectomy had been reached (6, 21). Thus, accurate and highly sensitive laboratory procedures will be needed to confirm the absence of *O. volvulus* infection. Antibody detection has been selected as an appropriate procedure (2, 17). However, since how long it takes until the anti-*O. volvulus* antibodies disappear from sera of treated patients is unknown, the presence of IgG and IgG4 antibodies was investigated in serum samples of people who had been under biannual treatment with ivermectin since 1991 in the zone of onchocercosis endemicity of Oaxaca, Mexico.

We developed and validated an ELISA for *Onchoerca* antibodies that can be used as a tool to evaluate the status of the
Mexican Onchocercosis Elimination Program in Oaxaca. ELISA-IgG4 not only gave higher serodiagnostic values than ELISA-IgG but also showed no cross-reactivity rates; in addition, the kappa coefficient index (0.78) indicated a good concordance between tests. Therefore, ELISA-IgG4 looks to be a useful test for detection of serum antibodies in onchocercosis infection. Mean values obtained in ELISA-IgG with sera from confirmed *O. volvulus*-infected patients were significantly higher than those obtained in the healthy, noninfected group from the area of endemicity, suggesting that IgG antibodies are the result of activation of the host’s immune response in infected individuals. The positive samples found (n = 3) in the endemic controls (group 2) could actually be individuals infected with *O. volvulus* but who are in the prepatent stage of the infection, as pointed out by Egwang et al. (8) and Rodríguez-Pérez et al. (18), justifying the finding that these subjects didn’t have *O. volvulus* microfilariae in their skin when they were parasitologically examined. It is possible that these positive cases probably pertained to people who, for whatever reason, have been capable of eliminating the *O. volvulus* microfilariae from their skin; thus, residual IgG antibodies could be detected. This comment is suggested by the fact that these samples were negative in the ELISA-IgG4, which is related to the concept that the presence of IgG4 isotype in serum is associated with an actual infection (5, 8, 13). Alternatively, positive results could be due to cross-reactions with antigens not tested here.

Epidemiological studies of onchocercosis have frequently reported a number of nonresponding individuals whatever the antigen or serological test used. Seronegative but actually infected individuals have been found by using several antigenic preparations in a number of serological tests (3, 4, 10–12, 16, 18). Thus, it was not surprising that in this work some samples from nonresponders to OvAw have been detected. Furthermore, scattered individual results showed that the intensity of the humoral immune response against OvAw antigens is clearly heterogeneous, whatever the Ig isotype searched. Similar results have been previously reported (4, 14).

Because no other human filarial infections exist in Mexico, we were not concerned about their reported cross-reactivity with crude antigen. Our data show that cross-reacting antibodies were only detected in samples from *T. spiralis*-infected patients and when total IgG was searched. This finding was not surprising because some epitopes present in antigens of *T. spiralis* muscle larvae have been identified by ELISA and Western blotting of serum samples from onchocercosis-infected patients (7, 19). However, there is scarce information regarding the prevalence of human trichinellosis inside areas of onchocercosis endemicity in Mexico; thus, the real impact of this cross-reactivity in antibody detection in this filarial infection cannot be estimated. Nevertheless, it is worth noting that the
obtained cross-reactivity was only detected when total IgG was searched; therefore, identification of the IgG4 isotype actually eliminated the problem.

The results presented herein suggest a significant impact by the actions of the Onchocercosis Elimination Program in Mexico on parasite transmission. This comment is based on the fact that IgG but not IgG4 isotype antibodies were found in three samples from individuals living in the Oaxaca focus of Mexico, which has been under biannual ivermectin treatment since 1991. In fact, although $A_{490}$ mean values obtained with these positive serum samples were high (0.835 ± 0.2 [mean ± standard deviation]), it is possible that the detected IgG antibodies could be the result of older O. volvulus infections, since these positive samples came from people 28, 34, and 78 years old. Furthermore, since no IgG4 antibodies were found in any of the serum samples from the putative onchocercosis group, the likelihood that diagnostic IgG4 antibodies disappear from sera of these kinds of individuals may be established between 5 and 10 years of treatment. This period of time is suggested because Rodríguez-Pérez et al. (18) reported that almost one-sixth of a round (5 years) of biannual ivermectin treatment. In addition, although the original serological status of the patients studied here is not available and the expected seroconversion could not be confirmed, the likelihood that IgG4 antibodies persist no more than 10 years in serum of ivermectin-treated individuals, as documented herein, suggests that detection of this Ig isotype in enzyme immunoassays may be used with high reliability as an auxiliary tool to confirm the interruption of the O. volvulus transmission in zones of endemicity under ivermectin therapy.

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