Evaluation of an OV-16 IgG4 Enzyme-Linked Immunosorbent Assay in Humans and Its Application to Determine the Dynamics of Antibody Responses in a Non-Human Primate Model of *Onchocerca volvulus* Infection

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Abstract. Onchocerciasis is a neglected parasitic disease targeted for elimination. Current World Health Organization guidelines for elimination include monitoring antibody responses to the recombinant *Onchocerca volvulus* antigen OV-16 in children to demonstrate the absence of transmission. We report the performance characteristics of a modified OV-16 enzyme-linked immunosorbent assay (ELISA) and describe anti-OV-16 responses in serum samples from laboratory-inoculated nonhuman primates (NHPs) in relation to microfilariae (mf) in skin snip biopsies. This OV-16 IgG4 ELISA had sensitivity and specificity of 88.2% and 99.7%, respectively, as determined by receiver operator characteristic analysis using a serum panel of 110 positive and 287 negative samples from people infected with other filariae or other parasitic infections. Anti-OV-16 responses in inoculated NHP ($N = 9$) were evaluated at quarterly intervals for IgM and the four IgG subclasses. Enzyme-linked immunosorbent assay results showed a well-defined IgG4 reactivity pattern and moderate IgG1 antibody responses. Meanwhile, the reactivity by IgG2, IgG3, or IgM did not show a clear pattern. Temporal evolution of IgG4 reactivity was evaluated through monthly testing, showing that NHPs developed anti-OV-16 IgG4 on average at 15 months postinoculation (range: 10–18 months). The average time to detectable mf was also 15 months (range: 11–25). The OV-16 ELISA used in this study was robust and allowed the detection of IgG4 responses, which were observed only among animals with detectable mf ($N = 5$), four of which showed declines in antibody responses once mf cleared. These findings also confirmed that the most informative antibody subclass responses to OV-16 are IgG4.

INTRODUCTION

Onchocerciasis, also known as river blindness, is a neglected tropical disease caused by the filarial parasite *Onchocerca volvulus*. The disease may lead to dermatitis, which can be debilitating, and visual impairment or blindness. The symptoms are the result of the host's inflammatory response to dying microfilariae (mf). The adult parasites do not cause debilitating symptoms and are localized in subcutaneous nodules that are typically found over bony prominences. Approximately 187 million people, most of whom live in sub-Saharan Africa, are at risk for infection with *O. volvulus*.¹ Humans are the only known definitive host for *O. volvulus*.^{2,3} Annual and biannual ivermectin (IVM) mass drug administrations (MDAs) have been shown to interrupt transmission in several American and African foci, and four countries have been verified to be free of onchocerciasis.^{1,4–13}

Elimination efforts have been challenged by inadequate treatment coverage, migration, and recrudescence of infections in areas of suspended treatment. Previous monitoring efforts have relied on the detection of mf in skin snips taken from the iliac crest.^{14–16} Although skin snip microscopy is a sensitive tool in hyperendemic or untreated populations where most infections result in high microfilaria loads, this assay has poor sensitivity in low-transmission settings where microfilaria loads are lower.^{17,18} Antibody-based assays for onchocerciasis use the recombinant antigen OV-16 of *O. volvulus* (OV-16 enzyme-linked immunosorbent assay [ELISA]). The OV-16 antigen is a recombinant phosphatidylethanolamine-binding protein,^{19–21} which was produced as a glutathione-S-transferase fusion

protein at the Laboratory of Parasitic Diseases, National Institutes of Health. This antigen that localizes to the hypodermis, cuticle, and uterus of female *O. volvulus*¹⁸ is regarded as a highly sensitive and specific antigen for detection of IgG4 in people with prior exposure to onchocerciasis.^{19,20} Detection of anti-OV-16 IgG4 is more sensitive and less invasive than skin snip microscopy.²² The anti OV-16 ELISA has been used to evaluate the status of disease transmission in the Americas^{6,7,23} and some parts of Africa.^{10,11,24}

A good understanding of the temporal evolution of the host immune response to *O. volvulus* infection is needed to be able to use OV-16 for monitoring onchocerciasis control and elimination efforts. The present study reports the refinement of an ELISA method using a panel of human serum specimens from onchocerciasis-endemic areas in Africa and from individuals with a variety of non-OV parasitic infections. The refined ELISA was used to characterize the antibody responses to OV-16 by IgM isotype and IgG subclasses. The characterization work used archived samples and parasitological data from nonhuman primates (NHPs) previously inoculated with infectious larval stages of *O. volvulus* and followed monthly for 2–5 years after inoculation.^{25,26}

METHODS

Ethics statement. The samples used in this study were drawn from reference collections at the Centers for Disease Control and Prevention (CDC), Division of Parasitic Diseases and Malaria (*O. volvulus*, *Wuchereria bancrofti*, *Schistosoma mansoni*, and *Strongyloides stercoralis*) or provided by the National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases (NIAID) (*Loa loa*), the NIH/NIAID Filariasis Research Reagent Resource Center (FR3, www.filariasiscenter.org, mainly for lymphatic filariasis), or the Navy Army Medical

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Research Unit 6 in Peru (for *Mansonella ozzardi*). The specimens from individuals with onchocerciasis were collected by CDC in collaboration with partners in Uganda and Ethiopia through protocols that were approved by the appropriate CDC (protocol no. 6196), Ugandan, and Ethiopian ethical review committees.

Samples and parasitological data from NHP were obtained from previously described studies conducted between 1987 and 1995 at the Yerkes Regional Primate Research Center in Atlanta, Georgia (Yerkes protocol numbers Y90/3/05 and Y91/2/06).^{25,27}

Enzyme-linked immunosorbent assay for OV-16. We refined an ELISA protocol previously used in evaluations for OV-16 reactivity in Guatemala.^{7,28} The following adjustments to that protocol were made: antigen concentration was reduced from 1.0 to 0.5 µg/well and the incubation temperatures were set to 37°C rather than room temperature. Furthermore, time to stop the ELISA development was optimized for accuracy based on the confidence intervals (CIs) for the upper and lower boundaries of the assay at 15, 30, 45, and 60 minutes.

The optimization of time to development used a standard curve consisting of 2-fold serial dilutions of a highly reactive reference sample, diluted from 1:200 to 1:12,800. ELISA optical density (OD) values from the standard curve were read at 15-minute intervals up to 60 minutes, and after the addition of 1N sodium hydroxide (NaOH) to stop the chromogenic substrate reaction. The resulting plots of the standard curve had a sigmoidal shape and were analyzed using the 5-point Hill's logistic regression model,²⁹ available in SoftMaxPro v 5.0 (Molecular Devices, San Jose, CA). The model provides information on the correlation of the curve, and parameters A, B, C, D, and E. For this analysis, we focused on parameters D and A, where D represents the value at the upper asymptote of the sigmoidal curve (upper dynamic range of the assay), and A represents the value of the lower asymptote (lower range of the assay). Parameter C is the calculated point of inflexion in the middle of the sigmoidal curve and B is the slope of the curve at point C. The fifth parameter of this equation is E, which reflects the symmetry of the curve. The values for D were used to determine upper dynamic range of the assay and the CI of D was used to evaluate the optimal time for substrate reaction, as it will reflect the range of certainty of the OD values obtained at different time points. Values and CI for A were used to confirm the lower dynamic range of the assay.

The optimal time for chromogenic substrate reaction of the ELISA was evaluated at four different times: 15, 30, 45, and 60 minutes, based on the CI of D using the 5-point logistic regression model. The time for substrate reaction was considered optimal if 1) values and CI of D were within the dynamic range of ELISA OD (0.0–3.99) and 2) the ratios of their CIs divided by the parameter (CI_D/D) were less than 10%.

A detailed protocol of the OV-16 ELISA is included as a Supplemental File.

Performance characteristics of the OV-16 ELISA. The performance of the OV-16 ELISA was characterized by receiver operator characteristic (ROC) analysis, using a panel of 399 human specimens. *Onchocerca volvulus*-positive specimens ($N = 102$) were obtained from people with patent infections, where 99 were confirmed by skin snip microscopy and polymerase chain reaction (PCR) and three by PCR alone. Negative samples ($N = 297$) were obtained from people living

TABLE 1
List and characteristics of the human specimens used to evaluate the performance of the OV-16 enzyme-linked immunosorbent assay

Country	Positive <i>Onchocerca volvulus</i>	Negative															Total									
		Filarial hematodes										STH		Other helminths				Protozoa		Negative						
		Wuchereria bancrofti (WB)	WB Pacific)	Brugia malayi	WB + Amoeba	WB + Ascaris	WB + hookworm	WB + Schistosoma	WB + Strongyloides	WB + Trichuris trichiura	Loa loa (returning travelers)	Mansonella ozzardi	Ascaris lumbricoides	Ascaris + Amoeba	Strongyloides	Enterobius		Hymenolepis nana	H. nana + Hymenolepis diminuta	Schistosoma mansoni	Amoeba	Amoeba + Giardia	Amoeba + H. nana	Endemic neg.	Onchocerca ochengi-endemic area	US negative
Argentina	—	—	—	—	—	—	—	—	—	—	—	—	—	—	26	—	—	—	—	—	—	—	—	—	26	
Bangladesh	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	
Brazil	—	—	—	—	1	1	—	—	2	—	—	2	5	—	—	1	3	1	—	12	1	3	10	—	46	
Haiti	115	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	115	
India	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	25	
Indonesia	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7	
Kenya	—	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	20	—	—	—	—	—	20	
Mali	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	
Peru	—	—	—	—	—	—	—	—	—	—	10	—	—	—	—	—	—	—	—	—	—	—	—	—	10	
Sri Lanka	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	14	
Tahiti	—	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	
Ethiopia	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8	
Uganda	94	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	94	
US	—	—	—	—	—	—	—	—	—	10*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	10	
Total	102	147	5	7	1	1	1	2	2	10	10	2	5	5	26	1	3	1	20	12	1	3	23	3	10	399

*Expatriate patients, U. S. National Institutes of Health.

in areas where *O. volvulus* is not endemic. Most of the negative samples ($N = 287$) were from persons living in nonindustrialized countries where onchocerciasis was not endemic; 261 had a parasite diagnosis other than onchocerciasis and 26 did not report a parasitic infection. The remaining 10 negative samples were from U.S. residents without history of onchocerciasis (Table 1).

The assay performance of the IgG4 ELISA for human sera and the identification of cutoff limits to maximize the specificity of the assay were determined using ROC analyses using the pROC package in R version 3.0.1³⁰ and SPSS Statistics V. 21 (IBM Corp., New York, NY). Sensitivity, specificity, and 95% CIs for area under the curve (AUC) were also calculated.

Evolution of anti-OV-16 antibody responses in NHP and patent *O. volvulus* infections. To better understand the isotype and IgG subclass responses from laboratory-induced infections, we used archived serum/plasma samples from nine laboratory-maintained adult chimpanzee (*Pan troglodytes*) NHPs from studies conducted between 1987 and 1995^{25,27} (Table 2). These studies focused on the evaluation of several species of NHPs, including chimpanzees, as potential models to understand the dynamics of human onchocerciasis.²⁵ In these studies, NHPs were inoculated with viable third larval stages (L3) of *O. volvulus* harvested from infected *Simulium* blackflies and followed up for 2–8 years. Monthly blood draws and detection of parasitological data of mf in skin, including a pre-inoculation sample, were performed throughout the studies. Leftover sera or plasma from the original studies were archived frozen at -80°C until used in this study. Because some NHPs received a single inoculum, whereas others were exposed multiple times to *O. volvulus* larvae (Table 2), only the five that were successfully infected with a single inoculum were selected to estimate time to seroconversion. Quarterly samples from the NHPs in this study were verified for immunoglobulin reactivity before OV-16 ELISA testing. The evolution of antibody responses was evaluated in relation to detection of mf in skin biopsies, a marker of patent infection. The existing data on mf detection on skin biopsies²⁵ were averaged and normalized to mf/skin snip/monthly sample.

RESULTS

OV-16 ELISA optimal development time. The optimal time to development for the OV-16 ELISA was evaluated by the 5-point logistic regression model on the data collected at 15,

30, 45, and 60 minutes (Figure 1). The fit of the data with the 5-point parameter curve had correlation values between 0.99 and 1.0, validating the use of this model. The ratio of $\text{CI}_D/D \times 100$ was smallest at 60 minutes of development (7.7%), indicating that the assay had increased precision at 60 minutes when compared with 15, 30, and 45 minutes of substrate development. Thus, 60 minutes was chosen for substrate development. The use of NaOH to stop substrate reaction at 60 minutes did not affect the CI (Figure 1). Values and CI for the lower limit of the assay were based on the A parameter and were in agreement with the lower limit optical densities for ELISA assays (Figure 1).

Performance characteristics of IgG4 OV-16 ELISA. The highest specificity was reached when the cutoff value was set to an OD value of 0.05. At this cutoff, the specificity was 99.7% with a sensitivity of 88.23% (Point B, Figure 2). This value was within the 95% CI of the value estimated by the program pROC (value A, Figure 2). The accuracy of the OV-16 ELISA was determined by the AUC of the ROC plot, with a value of 0.966 (95% CI: 0.938–0.993) (Figure 2).

Nonhuman primate infections with *O. volvulus*. Five of the NHPs developed patent infections, with overall first positivity at about 15 months postinoculation (PI) (mean 455 days, range 336–763 days). These five NHP remained mf positive for about 40 months (mean 1,212 days, range 700–1,602) (Figure 3), and three of those five NHPs still had detectable mf at the end of the study.

Antibody responses. The OV-16 ELISA antibody reactivity by IgM, IgG1, IgG2, IgG3, and IgG4 showed different patterns. The five NHPs that developed patent infections mounted anti-OV-16 IgG4 responses, with defined rise in OD values in the range of 1.04 and 3.87, whereas IgG1 antibody responses were less evident, with mild elevation in OD values (range 0.24–0.92). None of the infected animals produced informative temporal patterns of anti-OV-16-specific IgG2, IgG3, or IgM (Figure 4). By contrast, animals that failed to develop patent infections never produced OV-16-specific antibody responses despite multiple L_3 inoculations (Supplemental Figure 1).

Relationship of anti OV-16 IgG4 responses to mf. Monthly OV-16 IgG4 levels were compared with mf detection for the five NHPs that developed patent infections (Figure 3). On average, seroconversion (mean: 454 days PI, range 308–546) and first detection of mf (mean: 455 days PI, range 336–763) occurred at 15 months PI. However, seroconversion and patency did not show a defined sequence of occurrence.

TABLE 2
Detection of mf in skin snip biopsies from NHP through time in study

NHP no.	No. times inoculated	No. months mf+	No. months mf > 10/snip	No. days first mf+	No. days in study	Early withdrawal	Selected for seroconversion analysis
1	5*	25	1	640	2,752	No	No†
2	1	0	N/a	N/a	953	No	No‡
3	3	0	N/a	N/a	953	No	No‡
4	1	48	19	378	2,090	No	Yes
5	1	42	24	434	2,090	No	Yes
6	1	20	0	763	1,949	No	Yes
7	1	24	4	364	1,565	No	Yes
8	1	51	29	336	2,089	No	Yes
9	1	2	0	448	706	Yes	No§

mf = microfilariae; n/a = not applicable; NHP = nonhuman primate.

* NHP 1 was also exposed to 25 viable L_3 of *Onchocerca volvulus* at 987 days postinoculation using subcutaneous diffusion chambers.²⁷

† Not selected because of multiple exposures to viable larvae of *O. volvulus*.

‡ Not selected because of multiple exposures and lack of detection of mf at any time.

§ Not selected: withdrawn from the original study at day 706 because of pneumonia.

FIGURE 1. Values and confidence intervals (CIs) of parameters D and A at different times of substrate reaction (development) of the OV-16 enzyme-linked immunosorbent assay (ELISA), 5-parameter logistic curve.

In some animals, mf were detected before development of antibody reactivity and in other animals, antibody developed first (Figure 3). Four of the five NHP had a pattern of high OD responses over time. However, NHP 6, which also had a low level of microfilaridemia, developed only a low level of

fluctuating antibody reactivity. The four NHP with higher OV-16 reactivity had detectable OV-16 levels by the end of the study (44–66 weeks PI) (Figure 3) and three demonstrated a decline in OV-16 ELISA reactivity as microfilaridemia levels became low (NHPs 4, 5, and 7). In the case of NHP 6, this animal no longer had detectable anti OV-16 IgG4 at 42 months PI.

FIGURE 2. Receiver operator characteristics (ROC) plot for specificity and sensitivity of the OV-16 enzyme-linked immunosorbent assay (ELISA). The area under the curve (AUC) for the assay as performed was 0.966 (ideal test would have AUC = 1.0). Arrows indicate two cutoff points in the ROC plot: A—point determined by pROC with specificity: 98.3% (95% confidence intervals [CI]: 96.1–99.7) and sensitivity 92.2% and B—cutoff point selected for highest specificity within the 95% CI of point A, with an assay specificity of 99.7% (within the upper 95% CI of A) and sensitivity of 88.2%.

DISCUSSION

In this study, we optimized the performance characteristics of an OV-16 ELISA and used it to measure antibody responses in naturally infected humans and laboratory-inoculated NHPs. The ELISA used in this evaluation is a modification of the protocol currently used in several laboratories that conduct onchocerciasis elimination program evaluations to determine when to stop MDA. Those modifications, such as the use of defined incubation temperatures and a set time of 60 minutes for chromogenic substrate reactivity, were factors that improved the accuracy of this OV-16 ELISA.

The ELISA had high specificity (99.7%) and relatively high sensitivity (~88%) using a panel of *O. volvulus*-positive and *O. volvulus*-negative samples. Although this panel was similar in size to panels used to characterize other antibody-based assays for onchocerciasis,^{31,32} it could be further improved with samples from people having confirmed mono-infections with either *Mansonella streptocerca* or *Mansonella perstans*, which so far have only been obtained from areas co-endemic for onchocerciasis. Efforts are still ongoing to find those unique samples.

Assays used to inform program decisions about stopping MDA should have high specificity to minimize false-positive results. The 2016 World Health Organization guidelines state that fewer than 0.1% children should be OV-16 positive before stopping treatment, equivalent to less than two positive children in a sample of 2,000. Despite its high specificity, our

FIGURE 3. Monthly enzyme-linked immunosorbent assay (ELISA) OV-16 IgG4 reactivity of five nonhuman primates (NHPs) that were inoculated only once, in relation to average microfilariae (mf) per skin snip throughout the study period.

OV-16 ELISA must be used with care because the expected false-positive rate exceeds 0.1%.³³ This recommendation applies to all current assays for OV-16 serology as none of the previously reported assays has > 99.9 specificity. The development of a test or combination of tests that further augments

sensitivity while maintaining specificity of greater than 99% is still needed.

The evaluation of antibody responses in the NHP models provided important information. Detection of IgM reactivity was not as expected and did not appear to be linked to

FIGURE 4. Enzyme-linked immunosorbent assay (ELISA) anti-OV-16 reactivity of quarterly nonhuman primate (NHP) samples collected throughout the study period. Results are shown by each NHP and antibody evaluated: IgG1, IgG2, IgG3, and IgM. x axis indicates months postinoculation; the ELISA optical densities are plotted on the y axis.

inoculation with L3 or to be informative for serological monitoring or evaluation. Similarly, the assessment of IgG2 and IgG3 reactivity did not show detectable increments in OD after L3 inoculation. Minor increases in antibody reactivity were detected by the IgG1 subclass, but the most informative isotype was IgG4, where significant changes in antibody reactivity were visualized. These findings contradict previous studies that suggested IgG3 would be useful to detect *O. volvulus* infections,³⁴ although that testing was against whole parasite extracts. Our findings are consistent with qualitative analyses using Western blotting that showed the reactivity against low molecular weight antigens of *O. volvulus*, such as OV-16, was primarily with IgG1 and IgG4.³⁵

The availability of monthly skin snip data and serum for antibody evaluations allowed us to more precisely determine the time to seroconversion among NHP and the duration of detectable IgG4 antibody responses in relation to patency of infection. The 15 months to seroconversion that we report is longer than the previous reports of 16 weeks against whole worm extracts,³⁶ 7.5 and 13 months²⁵ against native antigens of 14 and 22 kDa, 7.6 months against either the 14 and 22 kDa native antigens, or the recombinant antigens OV-16, OC3.6, and OC9.3.²⁶ These differences may be due to the assays and antigens used as the earlier studies evaluated total IgG antibody responses against crude or partially purified extracts of *O. volvulus*.

Only NHP with patent infections developed IgG4 antibody reactivity to OV-16. The two NHPs that failed to develop patent infections (NHPs 2 and 3, Supplemental Figure 1) also did not develop OV-16-specific antibody responses, even after multiple inoculations. In addition, repeated exposures to L3, either through inoculations or exposure in subcutaneous chambers, did not result in significant anti-OV-16 antibody response

(NHP1, Supplemental Figure 1). The fact that seroconversion was only detected among NHP with patent infections suggests that exposures to L3 alone may not result in detectable IgG4 antibodies against OV-16.

These findings show that there is a significant time between infection and seroconversion. Thus, a seropositive person could have been inoculated with L3 of *O. volvulus* ≥ 15 months before seroconversion. This time lapse highlights the importance of proper posttreatment and post-elimination surveillance plans, and the complementary and important role of entomological assessments for onchocerciasis.

As expected, the duration of OV-16 antibody responses was longer than the period of infection patency. However, the ELISA values had a decreasing trend in four of the five NHPs that developed patent infections once mf declined. This finding is in agreement with a previous report of antibody responses of nonresident people with a history of long stays in onchocerciasis-endemic areas. In that study, 10 of 14 infected people who remained seropositive after microfilaria were no longer detected, and among the positives, seven had elevated anti-OV16.³⁷ In Nigeria, the comparison of OV-16-specific antibody levels among adults living in communities that had received annual IVM for about 17 years showed lower reactivity in 2009 when compared with baseline values from the 1990s, suggesting that some seroreversions had likely taken place.³⁸

In the NHPs, the IgG4 response was closely related to the detection of microfilaridemia and the decline in reactivity was associated with the disappearance of parasites. Although seroreversion was seen in one NHP, it occurred in the context of an infection that produced few microfilariae. Thus, it is unlikely that IgG4 antibody seroreversion could be used as a test of cure in an individual. However, monitoring population-level reactivity could be useful for determining the status of parasite transmission after community treatment is stopped. Investigating the rate of seroreversion in treated human populations, particularly in children born after MDA was implemented and transmission was suppressed, could add to our understanding of how best to use serology for long-term surveillance. In addition, and if properly calibrated into a validated method, serological data could be used to guide programs on the selection of their MDA strategies.

The key findings from this study are based on data from a small number of NHPs, which may not mirror the dynamics of infection and antibody responses among infected people in endemic areas. Despite this limitation, this study contributes to our understanding of the dynamics of antibody responses against OV-16, using an ELISA protocol that had robust performance characteristics.

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