Serum Immune Complexes as Diagnostic and Therapeutic Markers in Lymphatic Filariasis

Vandana Dixit,¹ A.K. Gupta,¹ P.S. Bisen,² G.B.K.S. Prasad,^{1*} and B.C. Harinath³

¹School of Studies Biochemistry, Jiwaji University, Gwalior, India

²Seedling Academy of Design, Technology, and Management, Jaipur, India

³Mahatma Gandhi Institute of Medical Sciences, Sevagram, India

In the present study we examined the utility of measuring antigen-specific soluble immune complexes (ICs) for the differential diagnosis and therapeutic monitoring of Bancroftian filariasis. Antigen-specific ICs were detected in significantly elevated levels in 90% of lymphatic filarial subjects suffering from disease manifestations, and very low levels in 8–10% of microfilaria (MF) carriers. The high incidence of antigen-specific ICs in the circulation of filarial subjects with clinical manifestations in contrast to the negligible presence in MF carriers facilitates serological

differentiation of these two important stages of lymphatic filarial infection. A sudden spurt in circulating levels of antigen-specific ICs was recorded in all MF carriers on diethylcarbamazine (DEC) therapy, and the magnitude of the increase correlated positively with the pretreatment levels of circulating MF. Thus, it is evident from the present study that circulating ICs (CICs) are potential serological markers for both the differential diagnosis of filarial infection and therapeutic monitoring of MF carriers. *J. Clin. Lab. Anal.* 21:114–118, 2007. © 2007 Wiley-Liss, Inc.

Key words: immune complexes; diethylcarbamazine; diagnosis; ELISA; serological marker

INTRODUCTION

Lymphatic filariasis caused by *Wuchereria bancrofti* is the second most important leading cause of permanent disability in India. The clinical manifestations of the disease range from acute symptoms such as adenolymphangitis and lymphedema (grade 1) to chronic manifestations such as hydrocele and elephantiasis. Diethylcarbamazine (DEC) is the most effective microfilaricidal drug for lymphatic filariasis. There is increasing evidence that DEC can reverse even acute clinical manifestations when administered over a long period of time. One of the important problems in the management of clinical filariasis is the lack of suitable laboratory tests to diagnose subclinical and clinical stages of filarial infection. Conventional parasite-based tests fail to diagnose subclinical and clinical stages of lymphatic filariasis. Although the detection of specific antifilarial antibodies in serum is useful for diagnosing clinical filariasis, it cannot differentiate microfilaria (MF) carriers from clinical filarial subjects, or exposed from uninfected individuals (1). Hence, there is a need for better diagnostic markers to diagnose acute and chronic filarial infections.

DEC remains the drug of choice for treatment of MF carriers. The therapeutic efficacy of DEC in control programs is conventionally assessed by monitoring the MF levels in night blood samples following drug therapy. The availability of a sensitive serological therapeutic marker would eliminate the shortcomings associated with night blood sampling for monitoring the efficacy of DEC therapy. In infected humans there is a continuous release of antigens into the circulation from living and dying parasites. This stimulates antibody synthesis and subsequent immune complex (IC) formation. Elevated levels of antigen-specific and nonspecific ICs in the serum of patients with lymphatic filariasis have been reported (3–5). Mehta and Khuller (6) used circulating ICs (CICs) as diagnostic and therapeutic markers in tuberculosis. The focus of the present study

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*Correspondence to: Dr. G.B.K.S. Prasad, SOS Biochemistry, Jiwaji University, Gwalior, India 474 011. E-mail: gbksprasad@hotmail.com

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was therefore to explore the utility of antigen-specific IC assays for the serodiagnosis of chronic lymphatic filariasis, and their application as therapeutic markers for assessing the microfilaricidal function of DEC.

MATERIALS AND METHODS

Sera

Intravenous or finger-prick blood samples were collected from the following groups of subjects living in regions of central India endemic for Bancroftian filariasis:

1. Microfilaremic group: Patients with MF in their peripheral blood (by night blood smear examination) and without any clinical manifestations.
2. Clinical filariasis group: Subjects who were amicrofilaremic and had clinical manifestations such as lymphedema grade 3, elephantiasis, hydrocele, etc.
3. Endemic normal group: Apparently healthy subjects who were microscopically MF-negative and had no clinical symptoms of filariasis or any history of filariasis.
4. Nonendemic normal group: Healthy subjects from Kashmir, Punjab, and Himachal Pradesh states (a region that is nonendemic for filariasis) served as non-endemic controls. The subjects selected for the study were from rural areas and had not traveled to filaria-endemic regions during the last 5-year period.

Night blood samples from all participants in the study were subjected to microscopic examinations for MF. Serum was separated immediately after blood collection and stored at -10°C with sodium azide (0.1%) as the preservative.

Collection of Whole-Blood Samples on Filter Paper

A total of 195 finger-prick blood samples from different groups of subjects were collected on Whatman 3 filter paper in multiple spots. The blood samples on filter paper were collected in such a way that they covered an area of 9-mm diameter (20 μL blood) as described by Malhotra et al. (2). The dried spots were stored in polyethylene bags (unsealed) at room temperature (when assayed the next day) or at 4°C (when assayed within 1 week of collection).

Elution of Blood Spots From Filter Paper

Elution of blood spots from filter papers was carried out as described elsewhere (7). In brief, the spots were cut out of the filter paper in small pieces with scissors and eluted in 1 mL of 0.05 M phosphate-buffered saline (PBS), pH 7.2, by continuous shaking for 2 hr at room

temperature. All of the eluates were centrifuged at 600g for 10 min to remove filter paper debris and were stored at -10°C with 0.1% sodium azide as the preservative. The filter paper eluant in 1 mL of buffer was assigned an equivalent serum dilution of 1:100 assuming a serum content in blood of 50%.

DEC Therapy

A total of 30 MF carriers were initially administered DEC at a dose of 6 mg/kg body weight for 14 days. Pretreatment blood samples were collected from all the 30 MF carriers to estimate antigen-specific CIC levels. Only 20 of the 30 participating MF carriers completed the full course of DEC therapy and provided filter paper blood samples three times (i.e., on day 7 of treatment, at the end of treatment, and 2 weeks after completing the full course of therapy).

Isolation of IgG Fraction From Clinical Filarial Serum

The sera of clinical filarial subjects who showed antibody to soluble microfilarial antigen in counter immune electrophoresis were pooled, and immunoglobulin-rich fractions were prepared by 33% ammonium sulfate saturation. The precipitated immunoglobulins were dialyzed against 0.01 M sodium phosphate buffer, pH 7.2, and the protein was estimated according to the method of Lowry et al. (7). The IgG fraction was isolated from the immunoglobulin fraction by DEAE-cellulose chromatography as described previously (3). The monospecificity of the separated IgG was confirmed by Ouchterlony's double immunodiffusion (8) employing goat-anti-human IgG (Bangalore Genei, Bangalore, India) and labeled as FSIg G. The preparation was stored at -20°C with 0.1% sodium azide until it was used.

Anti-C3 Enzyme-Linked Immunosorbent Assay (ELISA)

Conjugation of penicillinase (2.5 mg of powder with specific activity of 2100 U/mg protein; Sigma, St. Louis, MO) and FSIg G (24 mg) was carried out according to the method of Avrameas (9). The starch-iodine-penicillin substrate for penicillinase consisted of 150 mg of soluble starch in 27.5 mL of sodium phosphate buffer 0.2 M, pH 7.0, containing 10.64 mg of penicillin-V and 65 μL of 0.08 M of iodine in 3.2 M of potassium iodide solution. The substrate was prepared fresh for each use.

The optimum working dilutions of anti-C3 (1 ng/mL), sera (1:300), filter paper blood eluates (1:300), and FSIgG-penicillinase conjugate (1:1,000) were determined by checkerboard titration. An anti-C3 ELISA

was carried out as described previously (3). In brief, the wells in PVC microtiter plates were coated with 100 μ L of optimally diluted anti-C3 in 0.06 M of carbonate buffer, pH 9.6, by incubation at 37°C for 3 hr. The wells were coated with 3% BSA in carbonate buffer and washed with PBS/T. The second incubation was with serial dilutions of intravenous sera/filter paper eluates for 3 hr at 37°C. Following washing with PBS/T, the wells were incubated with 100 μ L of optimally diluted FSIgG-penicillinase conjugate. After thorough washing with PBS/T, the penicillinase activity in the wells was observed by incubation with 100 μ L of starch-iodine-penicillin substrate. Complete decolorization of the substrate as visualized by the naked eye was taken as a positive reaction. Statistical analysis was carried out with the use of COSTAT software.

The study design was approved by the institutional ethics committee, and informed oral consent was obtained from all of the subjects who participated in this study.

RESULTS

A total of 113 intravenous serum samples from the different groups were screened for filarial ICs by anti-C3 ELISA. The results are presented in Table 1. Three of 30 MF sera samples (median titer of 600), 28 of 30 clinical filarial sera (median titer 19, 200) showed antigen-specific immune complexes. One of 30 endemic normal sera and none of the non-endemic normal sera were positive for filarial ICs. An additional 195 finger-prick blood samples from the different groups were collected on filter paper and screened at an equivalent serum dilution of 1:300 by anti-C3 ELISA. The results are tabulated in Table 2. Fifty-four of 60 clinical filarial samples, five of 60 microfilaremic samples, and none of the endemic or non-endemic normal samples were positive for filarial ICs.

Only three of 30 MF carriers showed very low titers (<1,200) of CICs before DEC therapy. However, the circulating IC levels shot up severalfold with a median titer of 76,800 in all of the 20 MF carriers analyzed on the 7th day of drug therapy. Thereafter, a gradual

decline in IC levels was observed in all of the 20 MF carriers with a median IC titer of 19,200 when analyzed on day 15 (i.e., immediately after completion of drug therapy). A direct correlation ($P < 0.001$) between pretreatment levels of circulating MF and filarial IC levels was observed on day 7 of therapy (Table 3). Only six subjects were available on day 30 of DEC therapy for sample collection, and they showed a further decline in CICs with a reciprocal median titer of 4,800.

DISCUSSION

The ability to effectively diagnose the different clinical stages of filarial infection is important for both administering the appropriate drug regimen and monitoring the efficacy of drug therapy. Considerable efforts have been made to develop reliable diagnostic tests for lymphatic filariasis. A number of studies have reported the diagnostic utility of detecting filarial antigens in blood samples from MF carriers (10–13). Batteries of monoclonals raised against different filarial antigenic preparations were evaluated for diagnosing the microfilaremic stage of filarial infection. A sandwich ELISA employing OG4C3 monoclonal antibodies directed to the nonphosphorylcholine antigen of *Onchocerca gibsoni* was found to be sensitive on boiled sera from microfilaremic subjects (10,11); however, this monoclonal antibody failed with whole blood samples (13). A modified Immunochromatographic titer (ICT) test that

TABLE 2. Analysis of filter paper whole-blood eluates for antigen-specific immune complexes by anti-C3 ELISA

Group	Number examined	Number positive for IC	Percentage positivity
Nonendemic normal (MF negative)	30	0	0.0
Endemic normal (MF negative)	45	1	2.2
Microfilaremic (MF positive)	60	5	8.3
Clinical filariasis (MF negative)	60	54	90.0

TABLE 1. Antigen-specific immune complexes in sera of filaria endemic subjects

Group	Number examined	Median titer of CICs	Number positive for ICs
Nonendemic normal (MF negative)	23	0	0
Endemic normal (MF negative)	30	600	1 (3) ^b
Microfilaremic (MF positive)	30	600 (300–1200) ^a	3 (10) ^b
Clinical filariasis (MF negative)	30	19, 200 (1200–38, 400) ^a	28 (91) ^b

^aFigures in parentheses indicate range of titers.

^bFigures in parentheses indicate percent positivity.

TABLE 3. Correlation between pretreatment levels of circulating microfilaria and antigen-specific immune complexes in sera of microfilaria carriers on 7th day of DEC therapy

Subject code	Pretreatment levels of MF in microfilaremic subjects (number/20 μ L)	Reciprocal of filaria-specific CIC titer on 7th day of therapy*
1	1	4,800
2	2	4,800
3	2	4,800
4	3	4,800
5	3	4,800
6	5	76,800
7	6	76,800
8	6	76,800
9	8	76,800
10	8	76,800
11	9	19,200
12	10	76,800
13	10	76,800
14	15	76,800
15	17	19,200
16	20	76,800
17	22	76,800
18	27	307,200
19	28	307,200
20	30	307,200

*Significant correlation ($P < 0.01$) between pretreatment levels of microfilaria and CIC levels on 7th day of therapy.

utilizes whole-blood samples proved to be very rapid and sensitive for diagnosing only the microfilaremic stage of filarial infection (14). Although PCR-based diagnostic tests are sensitive, they have many limitations for large-scale applications (15). These monoclonal antibody- and PCR-based diagnostic assays are targeted mainly to microfilaremic subjects and are not promising for the diagnosis of subclinical and clinical stages of lymphatic filariasis. Antibody-detection assays were found to be less useful for serological differentiation between the microfilaremic and chronic stages of filarial infection (1).

Previous studies reported significantly elevated levels of ICs in the sera of clinical filariasis subjects (3,4). Although IC assays serve no purpose in diagnosing microfilaremic subjects, the present results (Tables 1 and 2) clearly document the diagnostic applicability of these assays for diagnosing the clinical stage of filarial infection. ICs at very low levels (reciprocal titer = 300–1,200) were detected in 8–10% of microfilaremic subjects prior to treatment. It would be interesting to conduct a follow-up study on IC-positive MF carriers prior to therapy and determine whether the microfilar-emia progresses toward the acute clinical filariasis stage over the course of time. IC measurement also appears to be useful for monitoring the pathogenesis of lymphatic filariasis; however, this hypothesis requires further validation.

The efficacy of DEC therapy is conventionally assessed by monitoring blood levels of MF following drug therapy. Previous studies on circulating antigen levels following chemotherapy revealed a transient increase followed by a gradual decline to base levels (16). Similar studies on specific antibodies reported a gradual decline in antibody levels following chemotherapy (2). The results of the present study indicate that the majority of MF carriers who were almost negative for CIC showed a steep rise ($P < 0.001$) in filarial-specific CIC levels soon after DEC administration. The degree of elevation in antigen-specific ICs following DEC therapy was directly proportional to the pretreatment levels of circulating MF (Table 3, $P < 0.001$). This observation clearly supports the notion that CIC measurements are useful for monitoring the therapeutic efficacy of DEC. The present study documents the diagnostic and therapeutic applications of measuring antigen-specific ICs in sera. In an earlier study we showed that filter paper eluates can be employed in ELISA for IC assays without loss of detectable sensitivity if used within 7 days of sample collection (17). The advantage of employing filter paper blood spots as diagnostic specimens in place of intravenous sera for measuring soluble ICs makes the assay system highly suitable for large-scale mass chemotherapeutic programs in lymphatic filariasis.

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