COURSE OF ANTIBODY PRODUCTION BY THE DIG-ELISA METHOD IN NEONATALLY INFECTED AND JUVENILE INFECTED RATS AFTER PRIMARY INFECTION WITH *BREINLIA BOOLIATI* (FILARIOIDEA : ONCHOCERCIDAE)

Beng-Chuan Ho, Lai-Ming Chew, Mulkit Singh and Eu-Hian Yap

Department of Microbiology, Faculty of Medicine, National University of Singapore, Lower Kent Ridge Road, Singapore 0511.

Abstract. The course of antibody production in Wistar neonatal and juvenile rats after primary infection with *Breinlia booliati* was studied by the DIG-ELISA technique using filter papers impregnated with capillary blood drawn from the infected rat tails at 7, 14, 28, 60 and 90 days post infection. Sera of neonatally infected rats did not react with adult worm antigen until day 7 and the titers of antibody remained at very low levels for the next 7 days. There was little tendency to eliminate the filarial larvae during this time. The antibody levels then rose rapidly throughout the next fortnight and increased to a maximum at day 60 after which the titer leveled out at a constant high value until early patency at day 90. On the other hand, antibodies could be detected in sera of juvenile infected rats as early as day 7 and the levels of antibody rose markedly to a maximum at day 28. During the period from day 60 to day 90 at early patency, the antibodies declined gradually to lower levels.

The humoral immune responses of 42 neonatally infected rats and 53 juvenile infected rats of 3 strains (Lewis, Wistar and Sprague Dawley) were tested against soluble *B. booliati* antigens from both female (1:50) and male (1:10) worm extracts by the DIG-ELISA method. Antibodies were detected in sera from all the microfilaremic and amicrofilaremic rats belonging to neonatally and juvenile infected groups. Sera of clean neonatal rats did not give a positive reaction zone.

INTRODUCTION

The diffusion-in-gel enzyme-linked immunosorbent assay (DIG-ELISA) and the diffusion-in-gel thin layer immunoassay (DIG-TIA) have been used for sampling and assaying techniques in mice experimentally infected with *Schistosoma mansoni* (Nilsson *et al,* 1985). The sensitivity of DIG-ELISA and DIG-TIA is comparable to that of the indirect hemagglutination (IHA), immuno-diffusion (ID) and enzyme-linked immunosorbent assay (ELISA) techniques (Nilsson *et al,* 1980b; Nilsson and Voller, 1982). The DIG-ELISA method is characterized by technical simplicity, high capacity and low cost which provide some advantages over currently available serological techniques. Furthermore, this technique gives qualitative as well as quantitative information with regard to antibody content (Nilsson *et al,* 1980a; Nilsson *et al,* 1980b).

In our previous studies we found that the infection of *Breinlia booliati,* a rat filaria, in three strains of laboratory white rats was dependent on both age and genetic-specificity (Ho *et al,* 1987). In these experiments, one group of rats was infected as neonates (less than 24 hours of age) while another group of 4-week old rats was injected with third-stage larvae of *B. booliati.* Among three strains of rats, the neonatally infected group of Lewis and Wistar strains exhibited a shorter prepatent period (85.2 days), higher infection rate (90-95%), higher recovery rate of adult worms (17-20%) and higher microfilaremia (log mean 3.1-3.3 mff/20 mm^3) as compared to longer prepatent period (99.2 days), lower infection rate (36.2-42.1%), lower recovery rate of adult worms (2-3%) and lower microfilaremia (log mean 2.1-2.4 mff/20 mm^3) in the juvenile infected group of the same rat strains. The third strain of Sprague Dawley rats was intermediate in susceptibility in *B. booliati* infection. It

was therefore of interest to employ the DIG-ELISA technique to compare antibody levels in various infected groups of the three rat strains.

A modification of the DIG-ELISA, using filter paper disc impregnated with blood or serum as source of diffusion was described by Nilsson *et al* (1985). Definitive advantage of this technique as pointed out by the authors was the possibility of analysing quantitatively antibodies in capillary blood and of storing sampled material for a considerable time. In the present study the antibody response of neonatally infected and juvenile infected rats of the Wistar strain were compared at various time intervals, commencing from the 7th day after the initial infection until the appearance of mature adult worms in the host.

MATERIALS AND METHODS

DIG-ELISA technique

Sera : Sera from white rats were obtained from 20, 17 and 4 neonatally infected rats of Lewis, Wistar and Sprague Dawley strains, respectively, and also from 17, 17 and 19 juvenile infected rats of the three corresponding rat strains, respectively. The detailed procedures of infection have been described previously (Ho *et al,* 1987). The blood samples were drawn from the heart of the infected rats 8 to 10 months after infection. Sera were stored at -20°C until use.

Antigens : a crude soluble extract of adult worms of *B. booliati* was prepared by homogenization of female and male worms separately in phosphate buffered saline (pH = 7.2). The homogenized worm suspension was then sonicated 3 times, 90 seconds at a time and then centrifuged at 12,000 *g* at 4°C for 3 minutes. The supernatant was dispensed into 0.5 ml aliquots and stored at -20°C for subsequent use. The total protein concentration of the antigen was estimated following the Lowry procedure (Lowry *et al,* 1951) using serum albumin as a standard.

Procedures for DIG-ELISA : the DIG-ELISA procedure for analysis of antibody content was performed as described by Elwing *et al* (1980). The results were evaluated by calculating mean (log) titers and standard deviation (SD).

Filter paper impregnated with capillary blood - DIG-ELISA technique

The immune response was studied on 10 each of the neonatally infected and juvenile infected rats of the Wistar strain by taking a series of blood samples during the time course of development of *B. booliati* at intervals of 7, 14, 28, 60 and 90 days after infection. A quantitative assay of antibody content was performed by means of the filter paper DIG-ELISA method as described by Nilsson *el al* (1985).

Sera were eluted by cutting 100 mm^2 discs through the blood spots and placing these into the wells of a tissue culture plate (Nunc, 12.5 × 8 cm). The serum was allowed to diffuse slowly in 0.08 ml PBS (pH = 7.2) for one hour. The antigen-antibody reactions using horse-radish peroxidase conjugate followed the technique of the standard DIG-ELISA procedure described above. The diameters of the yellowish reaction zones were measured and the geometric mean of antibody titers in the sera at each time interval was computed.

RESULTS

Comparison of antibody levels in the neonatally infected and juvenile infected rats by means of DIG-ELISA technique

Sera of the infected laboratory white rats of Lewis, Wistar and Sprague Dawley strains were obtained at 8-10 months after infection and the reaction of each serum was measured by the diameter of the reaction zones against extracts of both female (1:50) and male (1:10) adult worms, using DIG-ELISA technique. Protein concentrations of adult female antigen and adult male antigen were 14.75 mg/ml and 10.8 mg/ml respectively. Antibody titers to adult antigens in microfilaremic rats, amicrofilaremic rats with adult worms and amicrofilaremic rats which contained no adult worms were studied in 42 neonatally infected rats and 53 juvenile infected rats of 3 rat strains. The results obtained are presented in Tables 1 and 2. It can be seen that positive antibody reaction was demonstrated in all the infected rats.

Comparison of antibody levels among 3 rat strains : Table 1 shows that by using the female worm extract as antigen, there was no significant differ-

Table 1

Comparison of the reaction zone diameters of the DIG-ELISA test in neonatally infected and juvenile infected groups of 3 strains of laboratory white rats infected with *Breinlia booliati,* using female adult worm extract as antigen.

Source of sera	Rat strain		
	Lewis	Wistar	Sprague Dawley
	Geometric mean in mm^2 (No. of rats tested in parenthesis)		
Neonatally infected rats			
Infected rats having high microfilaremia (> 10,000 mff/20 mm^3)	5.22 ± 1.58^a (4)		–
Infected rats having moderate to low microfilaremia	7.55 ± 1.59^b (17)	8.50 ± 1.81^c (15)	11.85 ± 1.31^d (4)
Infected rats having no microfilaremia	5.59 ± 1.69^e (4)		–
Juvenile infected rats			
Infected rats having moderate to low microfilaremia	10.83 ± 1.66^f (3)	8.74 ± 1.80^g (6)	11.46 ± 1.30^h (5)
Infected rats having no microfilaremia	7.03 ± 1.66^i (14)	6.42 ± 1.48^j (11)	6.15 ± 1.28^k (14)
Amicrofilaremic rats without adult worms	6.33 ± 1.68^l (11)	6.75 ± 1.73^m (4)	6.25 ± 1.02^n (10)

t-test	*t*-test	*t*-test
Neonatally infected group among 3 rat strains	Juvenile infected group among 3 rat strains	Among microfilaremic and amicrofilaremic rats
p (a-b) = 0.10-0.20 NS*	p (f-g) = 0.50-0.70 NS*	p (b, c, d)-(f, g, h) = 0.20-0.30 NS*
p (a-c) = 0.10-0.20 NS	p (f-h) = 0.80-0.90 NS	p (b, c, d)-(i, j, k) = 0.02-0.05 S**
	p (g-h) = 0.30-0.50 NS	p (b, c, d)-(l, m, n) = 0.02-0.05 S
p (b-c) = 0.50-0.70 NS	p (i-j) = 0.50-0.70 NS	p (f, g, h)-(i, j, k) > 0.002 S
p (b-d) = 0.05-0.10 NS	p (i-k) = 0.30-0.50 NS	p (f, g, h)-(l, m, n) = 0.002-0.01 S
p (c-d) = 0.30-0.50 NS	p (j-k) = 0.70-0.80 NS	p (i, j, k)-(l, m, n) = 0.70-0.80 NS
p (a-e) = 0.80-0.90 NS	p (l-m) = 0.80-0.90 NS	
p (b-e) = 0.10-0.20 NS	p (l-n) < 0.90 NS	
p (c-e) = 0.20-0.30 NS	p (m-n) = 0.70-0.80 NS	
p (a-d) = 0.02-0.05 S**	*NS-not significant / **S-significant.	
p (e-d) = 0.02-0.05 S		

ence in antibody titers among sera from 3 rat strains. However, differences in the antibody response of 3 rat strains occurred when the male worm antigen was employed. As shown in Table 2, the antibody levels were apparently higher in both microfilaremic and amicrofilaremic rats of both neonatally infected and juvenile rats of Sprague Dawley strain (d, h, k) than those in the Lewis strain (b, f, i). Table 2 shows that antibody titers of microfilaremic neonatally infected rats of the Lewis (b) and Wistar (c) strains differed considerably. However, the difference in antibody levels was not so signifi-

Table 2

Comparison of the reaction zone diameters of the DIG-ELISA test in neonatally infected and juvenile infected groups of 3 strains of laboratory white rats infected with *Breinlia booliati,* using male adult worm extract as antigen.

Source of sera	Rat strain		
	Lewis	Wistar	Sprague Dawley
	Geometric mean in mm^2 (No. of rats tested in parenthesis)		
Neonatally infected rats			
Infected rats having high microfilaremia (> 10,000 mff/20 mm^3)		4.62 ± 1.33^a (4)	–
Infected rats having moderate to low microfilaremia	4.10 ± 1.52^b (17)	8.42 ± 2.08^c (15)	11.11 ± 1.08^d (4)
Infected rats having no microfilaremia		4.41 ± 2.03^e (4)	–
Juvenile infected rats			
Infected rats having moderate to low microfilaremia	5.82 ± 1.27^f (3)	8.22 ± 3.03^g (6)	11.77 ± 1.23^h (5)
Infected rats having no microfilaremia	4.15 ± 1.48^i (14)	5.52 ± 1.72^j (11)	8.59 ± 1.55^k (14)
Amicrofilaremic rats without adult worms	3.94 ± 1.53^l (11)	7.64 ± 1.63^m (4)	8.89 ± 1.64^n (10)

t-test	*t*-test	*t*-test
Neonatally infected group among 3 rat strains	Juvenile infected group among 3 rat strains	Among microfilaremic and amicrofilaremic rats
p (a-b) = 0.50-0.70 NS*	p (f-g) = 0.50-0.70 NS*	p (b, c, d)-(f, g, h) = 0.10-0.20 NS*
p (a-c) = 0.10-0.20 NS	p (f-h) = 0.002-0.01 S**	p (b, c, d)-(i, j, k) = 0.70-0.80 NS
	p (g-h) = 0.50 NS	p (b, c, d)-(l, m, n) > 0.90 NS
p (b-c) = 0.002-0.01 S**	p (i-j) = 0.10-0.20 NS	p (f, g, h)-(i, j, k) = 0.02-0.05 S**
p (b-d) > 0.001 S	p (i-k) > 0.001 S	p (f, g, h)-(l, m, n) = 0.001-0.002 S
p (c-d) = 0.30-0.50 NS	p (j-k) = 0.02-0.05 S	p (i, j, k)-(l, m, n) = 0.70-0.80 NS
p (a-e) = 0.80-0.90 NS	p (l-m) = 0.02-0.05 S	
p (b-e) < 0.90 NS	p (l-n) > 0.001 S	
p (c-e) = 0.10-0.20 NS	p (m-n) = 0.50-0.70 NS	
p (a-d) > 0.001 S	*NS-not significant / **S-significant.	
p (e-d) = 0.02-0.05 S		

cant in the juvenile infected rats of the same rat strains (f, g and i, j). It is interesting to note that as shown in Table 1 (using the female worm extract as antigen), there were no differences in the antibody titers in either microfilaremic and amicrofilaremic juvenile infected rats of Wistar (g, j) and Sprague Dawley (h, k) strains. However, by using the male adult extract as antigen (Table 2) the difference in antibody titers of the same strains of rats (g-h, j-k) became statistically significant.

Comparison of antibody levels between microfilaremic rats and amicrofilaremic rats of the neonatally infected and juvenile infected groups : There was no correlation between the density of microfilarias and antibody levels of the infected rats in 2 neonatally infected Lewis and Wistar strains. As seen in Tables 1 and 2, the antibody levels of 4 highly microfilaremic individual rats (a) and 4 amicrofilaremic rats (e) of the combined Lewis and Wistar rat strains were not significantly different from those of the infected rats of the same strains which had moderate and low microfilaremia (b, c). When the antibody levels of neonatally and juvenile infected groups of 3 rat strains pooled together were compared, immunoreactivity of the microfilaremic rats of the former group (b, c, d) was about at the same level as the microfilaremic rats of the latter group (f, g, h). An apparent difference, however, existed in the antibody levels between the microfilaremic rats (f, g, h) and amicrofilaremic rats (i, j, k) of the juvenile infected group and this was more significant in those amicrofilaremic rats which contained no adult worms (l, m, n). It is interesting to note that as shown in Table 2, the antibody levels of the microfilaremic rats of the pooled neonatally infected group (b, c, d) were not significantly different from those of the amicrofilaremic rats of the pooled juvenile infected group (i, j, k and l, m, n) against the male adult antigen. However, by using the female worm antigen, an apparent difference in antibody levels between the microfilaremic neonatally infected rats (b, c, d) and amicrofilaremic juvenile infected rats (i, j, k and l, m, n) was evident (Table 1).

Comparison of the time course of antibody production in neonatally infected rats and juvenile infected rats

The kinetics of the humoral immune response of neonatally infected and juvenile infected rats of the Wistar strain was determined by filter paper DIG-ELISA method using female worm extract antigen at the 7, 14, 28, 60 and 90 days after infection. The development of antibodies during these 5 time intervals is graphically demonstrated in Fig 1.

Time course of antibody production in the juvenile infected rat: An antibody level was first detected 7 days after the initial infection (log geometric mean = 0.08 mm^2 diameter) and the level increased slightly over the next 7 days. The antibody titer then grew very rapidly reaching the maximum at day 28 (log geometric mean = 0.90 mm^2 diameter), after which the titer decreased to a slightly lower level until day 60 (log geometric mean = 0.69 mm^2 diameter). During the later phase of prepatency and early patency at day 90 the antibody content of the sera of the same rats maintained at lower level (log geometric mean = 0.53 mm^2 diameter).

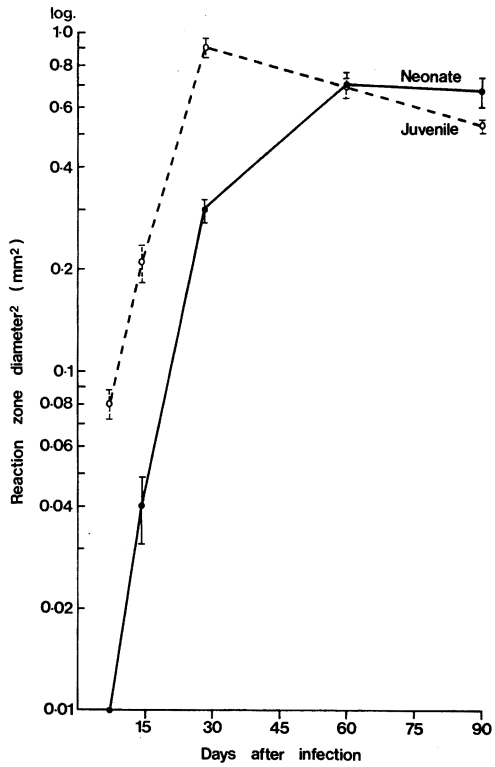

Fig 1—Levels of antibodies against *Breinlia booliati* in neonatally infected rats (●———●) and juvenile infected rats (○— — —○) of Wistar strain during course of primary infection. Circles (●, ○) and bars represent mean titers (± standard deviation) of sera tested by filter paper DIG-ELISA method.

Time course of antibody production in the neonatally infected rats

Unlike the juvenile infected rats, antibodies in the neonatally infected rats did not appear at day 7 and the antibody titer remained at a very low level at day 14 (log geometric mean = 0.04 mm^2 diameter). However, the antibody level rose rapidly

throughout the next fortnight (log geometric mean = 0.30mm^2 diameter at day 28). The antibody titer continuously increased steeply to a maximum at day 60 (log geometric mean = 0.70 mm^2 diameter) after which the titer levelled out at this constant high value until the early patency at day 90 (log geometic mean = 0.67 mm^2 diameter).

DISCUSSION

We know very little about the fate of filarial larvae from the time they are injected through the infective bite of arthropod hosts and disappear into the skin to the appearance of microfilaremia in the peripheral blood of the host. Sufficient sera are difficult to obtain from tiny neonates and young rats. One way to get around such problems is to employ the DIG-ELISA technique, using filter paper impregnated with the blood samples drawn from the tip of the rat's tail.

Gomez-Priego (1985) successfully applied the DIG-ELISA test to diagnose onchocerciasis by quantitative determination of serum antibodies from patients, using a soluble, crude extract from *Onchocerca volvulus* adults. The present studies have been made to explore the use of *B. booliati* female and male worm extract as antigens in DIG-ELISA test for detection of filarial antibodies in blood sera obtained from both neonatally and juvenile infected rats of 3 strains. It is interesting to note that by using the female extract as antigen, no significant differences in antibody titer among sera from 3 rat strains were observed (Table 1). However, differences in antibody response to the same 3 rat strains occurred when the male extract antigen was employed (Table 2). Our result showed that the antibody levels were apparently higher in both microfilaremic and amicrofilaremic rats of both neonatally infected (d) and juvenile infected (h, k, n) rats of Sprague Dawley strain than in those of the Lewis strain (b, f, i, l). The finding agrees with our previous observations that the rats of Lewis strain were more susceptible than the rats of Sprague Dawley strain to *B. booliati* infection (Ho *et al,* 1987). The differences of antibody titer between the infected rats of Wistar strain and those in Sprague Dawley and Lewis strains were inconsistent (Tables 1 and 2). The reasons for these variations are not very clear. Conversely, by using the female extract antigen (Table 1), an apparent difference existed in the antibody levels between the microfilaremic rats (b, c, d and f, g, h) and the amicrofilaremic rats (i, j, k and l, m, n). However, the antibody titers of the microfilaremic rats of the pooled neonatally infected group (b, c, d in Table 2) were not significantly different from those of the amicrofilaremic rats of the pooled juvenile infected groups (i, j, k and l, m, n in Table 2) when the male extract was used as antigen. The specificity of female and male worm extract in antigen-antibody complex is of great interest. Differences in immunogenicity of female and male antigens were shown by Mehta *et al* (1981) who found that the ability of the sonicated adult homogenates of *L. carinii* in inducing resistance in albino rats appeared to be due to the presence of microfilarial antigens in the extracts and not to adult antigens *per se.* They also found that sonicated adult male antigen was totally ineffective in inducing resistance to the infection in the same animal hosts. In contrast, Neilson (1978) using isoelectric focusing technique on somatic extract of adult worms and microfilariae of *D. viteae* indicated that the adult worms contained more antigenic components than the microfilariae. Specific characterization of the immune complexes of adult worms and microfilarial antigens may provide further insight into their application in the diagnosis of filariasis. Although it is desirable to measure the level of anti-larval antibodies in the sera of the rats using larval antigens, this however is not practicable because of problem inherent in recovering large numbers of developing larvae (Singh *et al,* 1976). Further studies have shown that there is a great deal of cross-reaction between different stages and species of filaria and one can detect anti-larval antibodies using antigens either from microfilariae or adult worms (Singh *et al,* 1980a; Singh *et al,* 1980b). Canlas and Piessens (1984) who analysed quantitatively and qualitatively of the reaction patterns of monoclonal antibodies of *B. malayi,* also indicated the existence of stage-specific antigens of *B. malayi,* as well of antigens shared by different stages of this parasite. Recently, Philipps *et al* (1986) also found that during the infection in cats, *B. pahangi* adult surface antigens cross reacted with epitopes present on earlier developmental stages.

In the present study, antibodies could be detected in the sera of juvenile infected rats of Wistar strain as early as 7 days after the initial infection with *B.*

booliati. For the following 7 days the titers remained at a very low level. On the other hand, the antibody titers in the neonatally infected rats only appeared at day 14. The antibody levels increased very rapidly throughout the next fortnight in both infected groups. However, the antibody titers at day 28 in the juvenile infected rats were three times (0.90 mm^2 diameter) that of the neonatally infected rats (0.30 mm^2). Exposure of the neonatal rats to filarial antigen appears to induce some tolerance. The immune system in neonatal rats is immature and hence tolerance may be induced more easily. This appears to be reflected in the lower peak response at day 28 in these rats. Spear *et al* (1973) studied the splenic lymphoid response at day 28 in these rats. They observed that among the splenic lymphoid cells in fetal and newborn mice the rate of increase in the numbers of all cell types (Ig-positive cells, O-positive cells and nucleated cells) in the spleen decreased during the first week after birth and reached a plateau during the second week. The increase of antibody to a significantly high level from day 14 to day 28 in the infected rats apears to be in accordance with the moulting process of the larvae. Singh *et al* (1976) had studied the course of development of *B. booliati* and found that moults of third-stage larvae to fourth-stage larvae and fourth-stage larvae to juvenile adult stage in the laboratory white rats took place on the 14th and 28th day respectively. During the later phase of prepatency and early phase of patency between day 60 to day 90, the antibody titers of both neonatally infected and juvenile infected rats declined slowly and remained at relatively high levels. It was evident from our present studies, as shown in Tables 1 and 2 that the antibody production in the microfilaremic rats of 3 pooled neonatally infected groups (b, c, d) was at the same level as that of the 3 pooled juvenile infected group (f, h, g).

Olson (1959) demonstrated that the migratory phase of third-stage larvae of *L. carinii* was the most crucial period for survival of the parasite in the white rats. The subsequent transferred fourth- and fifth-stage larvae which had previously developed in their natural host (the cotton rat) attained maturity in white rats. More recently, Weiss and Tanner (1981) studied the immunogenicity of the surface of larvae of *Dipetolonema viteae* in the hamster, suggesting that the excretory-secretory products during the moulting process from the third-stage to the fourth-stage larvae might be crucial for the stimulation of anticuticular antibodies. It is reasonable to suspect that since the initial titer of antibody in the juvenile infected rats was relatively high to begin with, a considerably large proportion of the third-stage larvae and migrating larvae of *B. booliati* would have been killed or retarded and eventually succumbed to the host immunity. On the other hand, antibodies produced by the neonatally infected rats at two weeks after infection were very low in titer and there was little tendency to eliminate the larvae from the host. If we take the percentage recovery of inoculated third-stage larvae of *B. booliati* for comparison, the mean recovery rate of the neonatally infected group of Wistar strain was about 3.4 times higher than that of the juvenile infected group of the same rat strain (Ho *et al,* 1987).

The other interesting finding in the present studies was the detection of anti-larval antibodies in the sera from the amicrofilaremic rats which harbored neither adult worms nor microfilariae (l, m, n in Tables 1 and 2) in their bodies at necropsy. Positive reactions were obtained in all the amicrofilaremic rats of 3 rat strains against both female and male worm antigens. It can be speculated that anti-filarial antibodies, presumably IgG or IgM, may persist long after the death of the larvae. This suggests that it may be possible to use DIG-ELISA technique to detect antibodies in amicrofilaremic patients with various clinical manifestations of filariasis.

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