Preadult Stage Parasites and Multiple Timed Exposure to Infective Larvae Are Involved in Development of Limb Edema in Brugia malayi-Infected Indian Leaf Monkeys (Presbytis entellus)†

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The pathogenesis of filarial limb edema is not known. The role of parasitological variables and parasite-mediated phenomena in the development of limb edema was investigated in the Presbytis entellus-Brugia malayi model. Infection was initiated with subcutaneous inoculation of infective third-stage larvae (L3), and the animals were reexposed to different doses of L4 at the prepatent, patent, and diminishing microfilaremia (0 to 5% of peak microfilaremia count) stages of infection. A large L4 inoculum size and repeated inoculation in the ankle region during the prepatent, patent, and diminishing microfilaremia stages of infection were found to be necessary for reproducible induction of limb edema. The preadult stage of the parasite was found to be the most potent inducer of limb edema, followed by L4 and L5. The presence of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-1β, and interleukin-6 in edema fluid in the leg receiving the parasite challenge indicated that the limb edema development was due to parasite-mediated cytokine responses. The absence of bacterial infection or anti-streptolysin O titer in the edema fluid and blood indicated that bacterial infection is not necessary for the development of limb edema.

Lymphatic filarial manifestations caused by Wuchereria bancrofti and Brugia malayi affect about 120 million people worldwide. The pathogenesis of the complex manifestations is not fully understood, because it is difficult to monitor the subjects from the time of exposure to infective third-stage larvae (L3) to development of chronic filarial manifestations. However, it is thought to involve at least three components: parasitological, immunological, and bacteriological. The contribution of each of these components is currently the subject of intensive studies. Epidemiological studies have shown positive correlations between lymphatic filarial infection and disease symptoms as development of symptoms follow a definite sequence, i.e., from infection to microfilaremia, then to amicrofilaremia, and finally to elephantiasis or hydrocele (2, 11, 12, 27).

Among the extrinsic factors that are thought to play a role in the development of filarial pathology, the most controversial are the secondary opportunistic bacterial and fungal infections. Exacerbation of acute and chronic episodes of lymphedema by opportunistic infections was reported to occur both in filarial patients (1, 12, 23, 24, 30) and in experimental animals (7). However, there are also reports that in many areas of endemicity where local hygiene is not maintained, there is no concurrent increase in pathology (30).

Although the basic information on immunological aspects of the filarial disease demonstrates differential immune responsiveness among various clinically positive individuals, the triggering or inducing factor(s) that leads to the development of clinical disease is not known. Studies with animal models have shown that the clinical and immunological outcomes of lymphatic filarial infection depend upon the frequency and intensity of exposure to the parasites. In cats, exposure to a single inoculation of Brugia pahangi induces lymphedema in about 15% of the exposed cats (6), whereas repeated small inoculations given at weekly intervals lead to the development of lymphedema in up to 40% of the animals (9). The Indian leaf monkey, Presbytis entellus, exposed to B. malayi develops systemic and local filarial disease manifestations, such as fever, eosinophilia, and episodic limb edema (20, 32), which closely resemble those in human patients and depend largely on the mode of exposure to infective larvae (21). In this model we found that disease manifestations developed during either low or no microfilaremia and that the humoral and cellular immune responses in certain symptomatic animals are different from those shown by animals that never developed any manifestations (8, 21). Also, some nonreactive parasite antigen molecules were detected in the sera of symptomatic animals, suggesting their possible involvement in the pathogenesis of manifestations. Further investigations were made with this model to explore the factors that contribute to the development of manifestations, particularly limb edema. The parameters considered were with a view to (i) develop an infection exposure protocol that increases the incidence of manifestations, (ii) ascertain the possible contribution of nonparasite factors in the development of disease manifestations and (iii) identify the parasite life stage(s) that may be involved in the development of limb edema. In the present study, we report (i) the effect of L4 exposure factors such as the size of inoculum and the site and timing of inoculations on the incidence of manifestations, (ii) studies on edema fluid for investigation of bacterial infection and inflammatory cytokines, and (iii) the induction of limb edema in infected and normal monkeys by
administration of extracts derived from various life stages of *B. malayi*.

**MATERIALS AND METHODS**

**Animals.** Young adult male Indian leaf monkeys (*P. entellus*), commonly known in Hindi as langur, 3 to 4 kg in body weight were obtained from local suppliers. Immediately on receipt, the animals were kept in quarantine for 45 days, during which time they were subjected to routine health check procedures, including clinical, biochemical, and hematological, and were thoroughly examined for tuberculosis (by the Mantoux test and chest X ray), intestinal helminthiasis (by examination of feces), and microfilaria (by night blood examination). Animals found positive for intestinal helminths were treated with mebendazole (Zodex; Concept Pharmaceuticals, Bombay, India) at 20 mg/kg orally for 3 days, which was repeated after 3 weeks. None of the monkeys was positive for *W. bancrofti* or *B. malayi* microfilaria. On completion of the quarantine and health check, the animals were transferred to the animal quarters of the experimental facility, where they remained under observation for not less than 4 weeks before the start of the study. Two days before the start of the study, the animals were again subjected to all of the tests described above, except those for tuberculosis, for a final health check. A total of 20 disease-free monkeys that were negative in all of the tests were finally selected for the present study. Throughout the research, and study periods the animals were housed in temperature (24 to 28°C)- and photoperiod (12 h of dark and 12 h of light)-controlled quarters protected from mosquitoes and other vectors by wire netting. The animals were fed on a commercial pellet diet (Nav Maharashtra Chakan & Oil Mills, Pune, India) supplemented with calculated quantities of bread, Bengal gram, and seasonal fruits and vegetables. They had free access to safe drinking water. Some monogamous pairs were kept under these conditions. Infected *A. aegypti* mosquitoes fed on microfilaria-infected monkeys through the subcutaneous route. The protein concentration of the extracts used was 1 mg in 0.5 ml. Each preparation was injected into both exposed and contralateral (unexposed) hind limbs at the ankle region. The inoculated areas were observed for development of manifestations if any, from 1 h after injection up to 8 h and thereafter at 24-h intervals for 7 days. Clinical assessment of the manifestations (i.e., edema and rectal temperature) was done until the disappearance of the manifestations as described above.

**Collection of edema fluid.** Edema fluid was collected from the edematous areas of the legs of the monkeys under aseptic conditions with the help of a heparinized syringe fitted with a 23-gauge needle. The fluid was stored at −20°C until use. The edema fluid from animals exposed to L_3_ was collected on three occasions: early stage (within 24 h of edema development), middle stage (the period when the volume of edema was maximum), and late stage (when the edematous reaction started receding). At each period the fluid was collected twice or thrice within 24 h. However, parasite extract-induced edema fluid was collected from animals when they showed well-developed pitting edema.

**Collection of blood and sera.** Sera were collected from symptomatic monkeys (during the period of manifestations), asymptomatic monkeys (which never showed any manifestations), and age-matched unexposed healthy monkeys 1 month after the first larval exposure and thereafter at monthly intervals until the termination of the experiment. The samples were stored at −20°C. Fresh heparinized bleed from these monkeys was collected and immediately subjected to bacteriological culture.

**Bacteriological examination in edema fluid and blood.** Edema fluids and freshly collected blood were cultured for the presence of aerobic bacteria (*Streptococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp. [gram positive]) and enterobacteria (*Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Aerobacter* spp., *Enterobacter* spp., *Proteus* spp., and *Pasteurella* spp. [gram negative]) and anaerobic bacteria (*Clostridium* group and anaerobic *Streptococcus*). For aerobic and anaerobic bacteria the techniques of Cruickshank et al. (4) and Wilson et al. (33) were generally followed. Briefly, blood or edema fluid in 0.1 ml was inoculated in duplicate nutrient broth tubes and blood agar and MacConkey lactose agar medium plates. The inoculum on the plates was distributed thinly by streaking it with a loop. The whole process was carried out under laminar flow aseptically. All of the tubes and plates were incubated aerobically at 37°C in the incubator and observed daily for 7 days. For the culture of anaerobic bacteria the tubes and plates were kept in McIntosh Fildes anaerobic jars with a GasPak and incubated at 37°C in the incubator. The plates and tubes were examined after 48 h for any growth.

For identification of bacteria after completion of incubation, one loopful of inoculum was streaked on blood agar and MacConkey agar plates and incubated both aerobically and anaerobically for 48 h at 37°C. Colonies appeared on the solid media after incubation, and a single representative colony was picked up from the agar plate and confirmed on the basis of its cultural and staining characteristics with Gram stain. Bacteria were identified on the basis of the morphology, motility, hemolysis on blood agar plates, and biochemical and sugar fermentation reactions were done according to the techniques of Cowen and Steel (3) and Cruickshank et al. (4).

**Measurement of ASO titer in edema fluid and serum.** The anti-streptolysin O (ASO) titer in both edema fluid and serum was determined by using commercially available kits (Orthodiagnostics, Mumbai, India) according to the method described by the manufacturer.

**Measurement of cytokine concentrations.** Interleukin (IL-6) and tumor necrosis factor alpha (TNF-α) (PharMingen) and IL-1β (Biosource International, Camarillo, Calif.) in edema fluids were measured by sandwich enzyme-linked immunosorbent assay with paired cytokine-specific monoclonal antibodies according to the manufacturer’s instructions. The concentrations of the cytokines were calculated from optical densities of samples versus optical densities of standards with known concentrations. Means and standard deviations of cytokine concentrations in two samples of edema fluid collected at each period were calculated.
RESULTS

Irrespective of the degree, site, and timing of L₃ exposure, all of the animals became microfilaremic between days 75 and 90 p.f.i. Microfilaraemia reached the maximum between days 90 and 180 p.f.i., followed by a sharp decline thereafter (data not shown). The level of microfilariae remained low after the peak microfilaraemia except in those animals which received L₃ during the period of diminishing microfilariaemia; in that case, the levels showed a marginal and transient rise within 30 to 60 days following reexposure to L₃ (data not shown).

Clinical manifestations. The incidence of limb edema in the monkeys exposed to L₃ is shown in Table 1. The episodic edema was the pitting and reversible type and was frequently associated with febrile attacks (102.5 to 104.6 °C). The duration of edema was 3 to 15 days. None of the monkeys of groups I, II, and III, receiving 250 or 500 L₃ in the groin or ankle region, developed edematous swelling in any part of the body. All eight animals of group IV, receiving inoculations in the ankle region, developed edema in the L₃-inoculated limb. The number of episodes of edema was five or six. The edematous attacks were associated with low or no microfilariaemia.

Bacteria and ASO titer in edema fluid and serum. Neither edema fluid nor blood of any of the monkeys showed any growth of aerobic or anaerobic bacteria. The ASO titer was negative in the edema fluid and sera of all of the infected or uninfected (control) monkeys.

Effect of administration of somatic extracts. Table 2 shows the development of edema in monkeys following inoculation of extracts derived from different life stages of the parasite. Venile adult worm extract, injected in the inoculated limbs of the animals which were first exposed to L₃ 50 to 60 days before, induced the maximum edematous reaction. Extracts of L₄ and L₅ stages produced less intense edematous reactions in the same category of animals. Microfilariae and adult worm extracts did not induce any visible edematous reactions. The same monches harboring infections of other ages failed to develop limb edema when administered any of the parasite extracts. The intensity of edema was categorized as mild, moderate, or severe at ratios of 1.01 to 1.15, 1.16 to 1.3, and above 1.3, respectively. Control limbs (contralateral limbs) of exposed animals and limbs of uninfected animals (which did not receive any L₃ inoculation) administered saline did not show any visible edematous reaction when injected with the parasite extracts.

Cytokine levels in edema fluid. Figure 1 shows the concentrations of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in edema fluids of limbs of monch (no. 20, 21, and 22) of group IV which developed spontaneous edema following exposure to B. malayi L₃, measured at three different time points, i.e., within 24 h of first appearance (early period), at maximum swelling (middle period), and when the edema started receding (late period).

In all three monches the patterns for the IL-1β concentration were comparable. In one of the three monches (no. 22), the concentration of IL-6 was same at all the time points. Monch 20 showed elevated levels of IL-6 during early development of edema, but at the middle and late periods the levels declined. In the edema fluid of the third monche (no. 21), the IL-6 concentration was highest during the middle period of edema development, but it dropped to very low levels by the late period. In two of the three monches (no. 21 and 22) the concentration of TNF-α in edema fluid at the early and middle time points was comparatively higher than that at the late time point. In contrast, in monch 20 this cytokine increased during the late period of the edema development compared to the other two periods.

In all three monches (no. 38, 39, and 53) receiving venile whole-worm extract through the subcutaneous route, of the three cytokines determined in the edema fluid during the late period of edema, the IL-6 concentration was highest, followed by IL-1β. The TNF-α concentration was the lowest (Fig. 2). These cytokines could not be determined in L₄ or L₅ extract-

### Table 1. Incidence and number of episodes of limb edema in Indian leaf monkeys (P. entellus) infected with B. malayi

| Group (n) | No. of animals | Inoculum | Edema episodes |
|-----------|----------------|----------|---------------|
|           |                | During prepatent period | During patent period | During diminishing microfilaria period | Total L₃ | Incidence (%) | No. |
| I 3       | 4 inocula of 25, 50, 75, and 100 L₃ | 250 | 0 | 0 | 
| II 3      | 4 inocula of 25, 50, 75, and 100 L₃ | 250 | 0 | 0 | 
| III 4     | 4 inocula of 125 L₃ each | 500 | 0 | 0 | 
| IV 8      | 3 or 4 inocula of 125 to 200 L₃ | 1 inoculum of 125 L₃ | 3 inocula of 100 L₃ each | 925 | 100 | 5 or 6 | 
| V 2       | Saline | | |
| a | Inoculations were given between days 1 and 45 p.f.i. in the groin region. |
| b | Inoculations were given within 8 days. |
| c | Inoculations were given in the groin region. |
| d | Inoculations were given at 13- to 15-day intervals in the ankle region. |
| e | Six animals received 125 L₃/dose × 4 and two animals received 125, 175 and 200 L₃/dose. |
| f | Inoculations were given in the ankle region. |

### Table 2. Effects of challenge with extracts of different B. malayi life forms on the development of edematous swelling in normal and L₃-infected Indian leaf monkeys (P. entellus)

| Group (n) | Reaction with the following somatic extract: |
|-----------|---------------------------------------------|
|           | Microfilariae | L₃ | L₄ | L₅ | Juvenile adult worm | Adult worm |
| Naïve (2) | − | − | − | − | − | − |
| Infected (6) | − | − | + | ++ | +++ | − |

a | Grading of edematous reaction (ratio of circumference between control and experimental limbs): −, no swelling; (1.0); +, mild swelling (1.01 to 1.15); ++, moderate swelling (1.16 to 1.3); ++++, severe swelling (more than 1.3). |

b | Animals harboring 50- to 60-day-old infections. |
induced edema due to insufficient edema fluid from the very low intensity of edema developed by the animals.

DISCUSSION

It is well understood that multiple acute attacks of adenolymphangitis lead to chronic irreversible deformity. However, it is not clear what causes more episodes in some individuals than in others. Is it the consequence of parasite burden, immunological reactions, bacterial involvement, or a combination of these or other factors? In our previous study we reported that 33 to 60% of Indian leaf monkeys (*P. entellus*) exposed to single or multiple doses of *B. malayi* L3 developed acute disease manifestations such as episodic limb edema, systemic symptoms of fever, and malaise. Earlier we also reported that 1 of the 12 symptomatic monkeys had developed limb edema that persisted for more than 10 months, with some signs of chronicity of the manifestations at late stage (8). In the present investigation we have tried to determine the parasitological variables responsible for the development of disease manifestations in this monkey model.

Five major factors appeared to be associated with the development of edematous swelling in the present model. First, repeated exposure of *P. entellus* to *B. malayi* L3 following the first larval exposure induced edematous swelling in the limbs of all (100%) of the animals. This occurred when animals were repeatedly exposed to different doses of L3 at the prepatent, patent, and diminishing microfilaremia stages of infection following the first larval inoculation. Using logistic regression analysis of the present and earlier data, we found that the incidence of edema development in *P. entellus* could be increased if L3 was injected between days 30 and 60 p.f.i. Limb edema was also induced by administration of somatic extracts of specific parasite life stages into the limbs of monkeys harboring 50- to 60-day-old infections. This finding suggested that repeated exposure during these periods perhaps was necessary for the development of edema in this model, as administration of the parasite extracts during other periods, such as between days 15 and 25, 150 and 160, or 280 and 290 p.f.i., failed to elicit any edematous reaction. Klei et al. (16, 17) have shown that jirds with *B. pahangi* infection developed a large number of lymph thrombi in the lymphatics between 60 and 90 days postinoculation in response to embolization of soluble somatic extract-coated cyanogen bromide activated Sepharose. This coincides with the time at which the female worms start releasing microfilariae. This evidence indicated that parasite-specific factors or products, reproductive products, or larvae or their metabolites might be responsible for the induction of edematous reactions. Several investigators have suggested that frequent and repeated exposure to infective mosquito bites positively correlates with infection prevalence, intensity of infection, and disease symptoms (11, 12, 14, 25). Our present findings, viewed against the background of reports in the literature, indicate that repeated and continuous exposure to parasites at the time when existing parasites are at molting...
stages or when female worms start releasing microfilariae might be necessary for the induction of edematous reaction.

Second, the most interesting finding of the present study is that the somatic extract of the preadult stage of the worm, when inoculated into the parasitized limb, evoked the most intense edematous swelling, whereas the extracts of L₂ or L₃ stages were much less effective. Further, the nonparasitized limb did not show any edematous swelling following administration of any of the worm extracts. These findings thus clearly indicate a decisive role of preadult stage parasite components in the development of limb edema. Recently, the lipopolysaccharides (LPS) of the endosymbiotic Wolbachia bacteria present in all the stages of the parasite have been considered to be potential stimulators of the inflammatory reaction (29). It is then likely that induction of intense edema in our study predominantly by the extracts of preadult stage is due to LPS of Wolbachia in the extracts. However, why the extracts of other stages injected in quantitatively identical amounts should fail to elicit identical edematous responses is not clear and remains to be investigated. Perhaps the density of the bacterial population in other stages is below the threshold required for initiating and sustaining the edematous reaction, but no information is presently available on this aspect. Nevertheless, the edematous reaction observed in the present model resembles those in patients after diethylcarbamazine therapy due to release of a large amount of parasite antigen and/or LPS-like molecule (13). With rodent models, Klei et al. (15) showed that prior sensitization of jirds with B. pahangi facilitated the development of an inflammatory reaction in tissues, but the identity of the parasite stage involved was not known.

Third, in the present study it was observed that a large inoculum size was required to induce an inflammatory (edematous) reaction, as a smaller inoculum (250 L₁) failed to induce such a reaction. In areas of endemicity a human subject acquires, on average, approximately 4,000 to 6,000 infective larvae through around 1,500 mosquito bites per year (10). It is thought that such repeated exposures to large numbers of larvae increase the chances for the development of adenolymphangitis. The site of L₂ exposure is an equally important determinant for development of an inflammatory reaction in tissues, but the identity of the parasite stage involved was not known.

Fourth, in the present study the absence of ASO titer or bacterial infection in serum or edematous fluid of monkeys indicated that bacterial infection was not involved in the development of acute episodic attacks of limb edema. This finding agrees with the report of Taylor and Turner (30), who considered the role of bacterial infection in enhancing lymphatic pathology to be controversial.

Finally, the presence of significant amounts of local inflammatory cytokines in the edema fluid of our monkeys indicates that cytokines play an important role in the development of limb edema. Rao et al. (26) found that infection of immunodeficient mice with Brugia species results in development of lymphedema and is associated with production of proinflammatory cytokines IL-1, IL-6, TNF-α, and granulocyte-macrophage colony-stimulating factor in lymph fluid of parasitized dilated lymphatics. They suggested that the regulatory activity of a network of these localized cytokines might cause the lymphatic lesions. In the present study we could demonstrate the cytokines directly in the edema fluid. Also, peripheral blood mononuclear cells from our infected monkeys (group IV), when stimulated with B. malayi antigen, produced significant amounts of IL-1β, IL-6, and TNF-α, of which the level of IL-6 was the highest (data not shown). In vitro and in vivo studies with filaria-infected animals and humans have shown that filarial parasites could stimulate production of IL-1β, IL-6, IL-12, TNF-α, and granulocyte-macrophage colony-stimulating factor, and they correlated with the development of acute and chronic disease manifestations (5, 13, 19, 29, 31; P. F. Turner, K. A. Rockett, H. Francis, K. Awadzi, E. A. Ottesen, and A. Clark, Abstr. Meet. Aust. N. Zealand Soc. Parasitol., 1992; M. Yazdanbakhsh, L. Duyun, L. Aarden, and F. Partono, Letter, J. Infect. Dis. 166:455–454, 1992). Regarding the identity of the parasite products or factors stimulating the cytokine production, not much is known at present, but they may include, as mentioned above, the LPS of the endosymbiotic Wolbachia spp. (29).

In conclusion, the present findings indicate that following first larval exposure, the mode of subsequent larval exposure, such as the size, site, and timing of exposure, plays a decisive role in the development of manifestations in the Indian leaf monkey model. The preadult stage appeared to be predominantly involved in evoking an acute edematous reaction in the infected monkeys, and the edematous reaction developed only in the limb that was challenged with parasite extract. The presence of inflammatory cytokines in systemic and edematous fluids suggests that the development of edema is evidently mediated by cytokines produced during the interaction between the host and the parasite or its products. The present study also shows that bacterial infection is probably not necessary for the development of acute episodic attacks of limb edema in the monkey model.

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