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* infection model. Infection was initiated with subcutaneous inoculation of infective third-stage larvae (L3), and the animals were reexposed to different doses of L3 at the prepatent, patent, and diminishing microfilaremia (0 to 5% of peak microfilaremia count) stages of infection. A large L3 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.
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 microfilariae (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 microfilariae. On completion of the quarantine and health check, the animals were transferred to the animal quarters of the experimental flarialiasis wing, 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 present study 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.

Infection. Twenty male langurs were divided into four groups and inoculated with infective L1 of B. malayi. The L1 were obtained from laboratory-bred female Aedes aegypti mosquitoes fed on microfilariaemic Mastomys coucha as described previously (22). Three animals each in groups I and II received a total of 250 L3 in the groin or ankle region in four divided doses within a span of 8 days. Animals in group III (n = 4) received 500 L3 in four divided doses spread over a period of 45 days. In these animals the inoculations were given in the groin region. Group IV, consisting of eight animals, received inoculations in the ankle region. An initial inoculum of 500 L3 in three or four divided doses (125 to 200 L3/dose) at 13- to 15-day intervals was given to each animal (six animals received four doses of 125 L3, and two animals received doses of 125, 175, and 200 L3), followed by a single inoculum of 125 L3, during patency and one to three inocula of 100 L3 during the period of diminishing microfilariaemia (we use the term diminishing microfilariaemia stage to indicate the period after patency during which microfilarial counts fell to and remained at 0 to 5% of the peak count). All of the animals received inoculations through the subcutaneous route. Animals in group V (n = 2) received saline in the ankle region subcutaneously and served as controls.

Microfilariaemia of infected animals was monitored between 9 and 10 p.m. by the membrane filtration technique (20) on days 60, 75, and 90 after the first L3 inoculation (p.f.) and thereafter at monthly intervals until the end of the study.

Clinical examination. All of the monkeys were examined daily for externally visible inflammation in the limbs. Rectal temperature was recorded on every alternate day. Quantitative assessment of swelling of limbs was made as described earlier (32). Briefly, peripheral (circumference) measurements were taken of the affected limb at three representative sites showing the lowest to maximal visible swelling between the knee and the ankle. The locations of these sites were determined in terms of distance from the knee joint towards the ankle, and measurements were then made at the same locations of the unaffected limb of the same animal. The swelling ratio was determined by dividing the sum of the three measurements for edematous limb by the sum of the three measurements for the unaffected limb of the same animal. The ratios were categorized into three range classes representing mild (ratio, 1.01 to 1.15), moderate (ratio, 1.16 to 1.30), and severe (ratio, more than 1.3) edematosus swelling. Any inflammation in the toes was also considered.

Preparation of parasite whole-worm extracts. Microfilariae, L3, L4, and pre-adult and adult worms were recovered from the peritoneal cavities of experimentally infected jirds (Meriones unguiculatus). L3 were isolated from B. malayi-infected A. aegypti mosquitoes fed on microfilaria-positive M. coucha 9 or 10 days before. Soluble somatic extracts from all life stages of the parasite were prepared aseptically as described in detail (20) and stored at −20°C. Briefly, the worms were washed thoroughly with sterile 0.01 M phosphate-buffered saline (pH 7.2) and homogenized in a Potter Elvehjem tissue grinder (A. Thomas Scientific, Philadelphia, Pa.) at 4°C. The homogenate was sonicated on ice at 20 kilocycles per s for 20 s, and 9 or 10 such strokes were applied to make the extracts homogenous. The protein content was measured by the method of Lowry et al. (18), and the antigen was stored in aliquots of 0.5 ml at −20°C until use.

Administration of extracts derived from various life stages of the parasite to monkeys. Animals of group IV harboring various stages of infection (between days 15 and 25 [prepatent], 50 and 60 [prepatent], 150 and 160 [patent], and 280 and 290 [period of diminishing microfilariaemia] p.f.) were used in this study. In order to find out which stage of the parasite extract has the potential of inducing manifestation, extracts of various life stages of the parasite or saline were administered in the normal and flaria-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 L3 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 blood from these monkeys was collected and immediately subjected to bacteriological culture.

Bacteriological examination in edema fluid and blood. Edema fluid 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 characters 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.
TABLE 1. Incidence and number of episodes of limb edema in Indian leaf monkeys (P. entellus) infected with B. malayi

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Inoculum</th>
<th>Edema episodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>During prepatent period</td>
<td>During patent period</td>
<td>During diminishing microfilaria period</td>
</tr>
<tr>
<td>I</td>
<td>3</td>
<td>4 inocula of 25, 50, 75, and 100 L3 Each</td>
<td>250</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>4 inocula of 25, 50, 75, and 100 L3 Each</td>
<td>250</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>4 inocula of 125 L3 Each</td>
<td>500</td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>3 or 4 inocula of 125 to 200 L3</td>
<td>1 inoculum of 125 L3</td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>Saline</td>
<td>Saline</td>
</tr>
</tbody>
</table>

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 Inoculations were administered between days 1 and 45 p.f.i. in the groin region.
f Inoculations were given in the ankle region.

RESULTS

Irrespective of the degree, site, and timing of L3 exposure, all of the animals became microfilaricemic between days 75 and 90 p.f.i. Microfilariaemia 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 microfilariaemia 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 microfilariae.

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 uninjected (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. Juvenile 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 L3 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 monkeys 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 monkeys (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 monkeys the patterns for the IL-1β concentration were comparable. In one of the three monkeys (no. 22), the concentration of IL-6 was same at all the time points. Monkey 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 monkey (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 monkeys (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 monkey 20 this cytokine increased during the late period of the edema development compared to the other two periods.

In all three monkeys (no. 38, 39, and 53) receiving juvenile 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-
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 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 microfilaria 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 L₃ or L₄ 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 parasitic 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 L₃) 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 L₃ exposure is an equally important determinant for development of an inflammatory reaction, since the monkeys inoculated with L₃ in the ankle region developed edematous swelling while those inoculated in the groin region did not. Three features related to the site of disease development in malayan filariasis are that (i) the lower extremities of the human body are the most preferred biting sites for B. malayi-carrying mosquitoes, (ii) leg edema is common, and (iii) B. malayi has a special preference for residing in the popliteal lymph nodes. In the present study too it was found that when the forearm instead of the hind limb was used for inoculation of L₃, subsequent challenge of the same forearm with parasite extracts failed to induce an edematous reaction in the arm (data not shown), indicating that the axial lymph nodes are not the preferred abodes for the parasite. These findings suggest that possibly all of these combinations of elements of the exposure profile may not occur uniformly in all subjects in areas of endemicity and hence only a certain percentage of the population, which has such a schedule or combination of exposures, shows manifestations of the disease.

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 immuno-deficient 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. 166:453–454, 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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