Lepr Rev (1985) **56,** 279–290

Viability of *Mycobacterium leprae* within the gut of *Aedes aegypti* after they feed on multibacillary lepromatous patients: a study by fluorescent and electron microscopes

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Accepted for publication 23 May 1985

Summary This paper describes the viability of *Mycobacterium leprae* within the gut of mosquitoes after they have bitten bacilliferous lepromatous patients. In the test experiments prestarved female *Aedes aegypti* were allowed to feed on bacilliferous leprosy patients, while in the control experiments mosquitoes were fed on a glucose-water-lepromin mixture containing dead *M. leprae*. The insects were sacrified for 7 consecutive days, their guts were dissected out and excreta were collected. These preparations were mounted and examined for acid-fast bacilli (AFB) by: 1, fluorescent staining technique; 2, by a light microscope using acid-fast staining technique; and 3, also by scanning as well as transmission electron microscopes. AFB were found in the gut and also in excreta but more abundantly in the earlier days after blood meal.

The fluorescent staining technique showed that AFB within the gut of mosquitoes became non-viable (red stain) after 4 days of blood meals. It also demonstrated multiplication of the viable bacilli (green stain) during early days. It was further observed that most of the solid bacilli quickly became granular and non-viable (red stain). Ultrastructural studies confirmed these findings and demonstrated membrane bound dividing bacteria within the gut of the insects mostly within 72 h after a blood meal. No such cell-division was found in the gut of mosquitoes artificially fed on the glucose-water-lepromin mixture.

These data together with light microscopic findings lend support to the transient multiplication of viable *M. leprae* within the gut of the mosquitoes after the bacilli were taken up from the circulation of lepromatous patients at least during the early period following a blood meal. However the possibility of transmission of the illness into humans by mosquito bites seemed to be remote,

0305-7518/85/056279+12 $01.00

because of the short viable time, quick fragmentation and elimination of the ingested bacteria from the gut of the insects.

Introduction

Acid-fast bacillaemia has been demonstrated in untreated borderline or lepromatous leprosy patients.[1] Laboratory bred mosquitoes (*Culex fatigens*), previously allowed to feed on lepromatous leprosy patients, often contain leprosy bacilli,[2] which was transmitted into mouse foot-pad[3] and some remained viable up to 48 h after the blood meal.[4] Thus in some parts of the world these arthropods might play a significant role in at least a mechanical transport of *M. leprae*. However, there is a paucity of data regarding the alterations in the morphology that might take place in *M. leprae* within their gut. Also their exact survival period within the insect's intestine remains to be determined. The present work is an attempt to find out: 1, whether there is any multiplication of the ingested bacteria; 2, to determine their survival time within the gut of the mosquitoes; and 3, also to document the ultrastructural changes occurring in *M. leprae* within their gut till they are excreted. These results have been compared with control experiments in which the mosquitoes were allowed to feed on a mixture of glucose, water and lepromin (containing non-viable *M. leprae*) in a proper proportion.

Materials and methods

A BREEDING OF MOSQUITOES IN STERILE CONDITION*

Adult female *Aedes aegypti* mosquitoes were obtained from a laboratory bred mosquito colony, kept at the Malaria Research Centre, Indian Council of Medical Research, Delhi.

B HUMAN MATERIAL

Ten (9 males and 1 female) leprosy patients including 9 LL and 1 maculo-anaesthetic case volunteered for the feeding experiments. Their age varied from 20 to 42 years. The duration of their illnesses ranged from 1 to 7 years. Diagnosis was based on clinical examinations and histological classification.[5] All cases except one 22-year-old patient with maculo-anaesthetic (MA) type of leprosy

* Filter paper containing desiccated mosquito eggs was kept in alcohol for a few minutes, and thereafter floated in 0·5 l of sterile distilled water, covered with a sterile net and kept at 28°C in a sterile chamber. The eggs were hatched next day; 50 units of neomycin sulphate and 50 units of polymyxin B sulphate were added. Every alternate day autoclaved dog biscuit and yeast were given as food. pH was maintained at 6·5 by changing water every third day. Fresh antibiotics were added when the water was changed. Pupa and adult mosquitoes were formed.

were on antileprosy treatment at the time of the study. Bacillary index was estimated, which varied from +2 to +4. One of the authors (KS) volunteered for the feeding experiment. He was taken as a control.

C FEEDING EXPERIMENTS PERFORMED ON BACILLIFEROUS LEPROMATOUS PATIENTS OR HEALTHY VOLUNTEERS

(1) A cage (8″ × 8″ × 8″) made of iron wires was covered on all sides by mosquito net cloth. In a typical experiment 40–50 prestarved female mosquitoes were taken. There was an opening through which the volunteers introduced their hands. The mosquitoes were starved for 12–24 h and then allowed to bite on the hands for 5 to 10 min during the day-time.

(2) The biting experiment was also performed with the apparatus described in Figure 1. The advantage of this apparatus is that the mosquitoes could be allowed to feed on a particular skin lesion.

After the blood meal mosquitoes were kept in the cage with proper humidity. A sugar source (raisins soaked in water or cotton wool soaked in glucose-water) was offered to the insects.

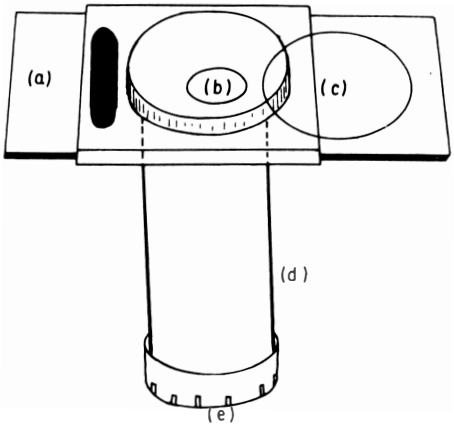

Figure 1. A plastic cylinder (d) had wire netting at one end (e) and a sliding stage with one opening at the other end (a). The mosquitoes were introduced through the opening (b) and thereafter it is closed. Before the biting experiments, the cylinder was put on the exposed skin of the volunteer, with the net (e) touching the skin. The mosquitoes bite the volunteers through the net (e). After biting the mosquitoes are taken out through (c) for dissection.

D FEEDING OF MOSQUITOES ON A GLUCOSE-WATER-LEPROMIN MIXTURE

Two volumes of 5% glucose solution were mixed with 1 volume of armadillo derived lepromin (World Health Organization) containing 4×10^7 bacilli/ml. This mixture was soaked in cotton wool and was kept on the mosquito cage net for 1–2 h and thereafter replaced by a plain pad containing glucose water. Sterile precautions were taken in the preparation of the mixture as far as possible.

E COLLECTION OF EXCRETA FROM MOSQUITOES

The excreta was collected from the glucose-water pad, mounted in a drop of phosphate buffer and stained for AFB.

F DISSECTION OF THE GUT OF THE MOSQUITO AND PREPARATION OF SAMPLES FOR THE DEMONSTRATION OF ACID-FAST BACILLI

Dissection of midguts was done according to the method described by Vanderberg & Gwadz[6] and they were examined for normal bacterial flora.*

Two to four fed mosquitoes were etherized at 24-h intervals and thereafter up to 7 days. The proboscii was cut and the guts were dissected out under an ordinary dissecting microscope by using 2 needles, in a drop of normal saline on a microscopic slide. The samples thus obtained were processed for light and electron microscopy. Excreta were collected from the water pad and were smeared either on microscopic slides or on grids for electron microscopy.

G STUDY OF THE VIABILITY OF MYCOBACTERIAL CELLS BY FLUORESCENT STAINING PROCEDURE

The method described by Kvach & Veras[7] was followed. In brief, 5 mg fluorescein diacetate (FDA) (Sigma) per ml acetone was prepared. 2 mg ethidium bromide (EB) (Sigma, USA) was dissolved in 1 ml Hanks' balanced salt solution (HBSS), pH 7·4, containing 0·05% Tween-80. A fresh working solution of FDA/EB was prepared by diluting the above FDA solution tenfold in acetone. A 0·02 ml volume of the diluted FDA was added to 5 ml HBSS containing 0·05% Tween-80. A 0·01 ml of EB stock solution was added to 5 ml HBSS containing FDA. The fixed smears of mosquito guts were stained with FDA/EB, incubated at room temperature for 10 min, wet mounted, sealed and seen under a Leitz microscope with filer BP-390 at the excitation and filter 2 at the barrier. Green cells were considered to be viable and the red stained bacteria were considered to be dead.

H LIGHT MICROSCOPY

The gut and excreta smears were stained by Ziehl–Neelsen stain, examined under an oil immersion objective of a Leitz light microscope and documented photographically.

* Mosquitoes were etherized, guts were dissected with sterile needles under a dissecting microscope in an inoculation chamber. These tissues were teased out in a drop of sterile saline, smeared on glass slides and stained with Gram stain. Part of the tissue suspension was cultured on blood agar and McConkey plates. No Gram positive or Gram negative bacilli were seen or grown.

ELECTRON MICROSCOPY

(1) *Scanning electron microscopy*. The intact guts from control-unfed, control-fed, patient-fed mosquitoes were sonicated in alcohol at 30 kHz frequency for 30–45 s. The sonication time was standardized by sonicating the gut tissues for different time durations (15, 30, 45 and 75 s). Sonication for a longer time completely smashed the gut as well as the bacteria.

The intact sonicated as well as teased guts of mosquitoes fed on patients and a control were mounted on polished aluminium discs with a very fine layer of quickfix, examined under a JSM 35 scanning electron microscope with a resolution power of 60 A and the results were documented.

(2) *Transmission electron microscopy*. Blood was taken from the gut of the mosquito, at 24 h intervals for 96 h, diluted 1:4 in phosphate buffer (pH 7·0) on microscopic slides. Formvar-coated grid (300 mesh) was floated upside down on blood-buffer mixture and the grid was placed on a moist filter paper and a drop of freshly prepared solution of 1% uranyl acetate was placed on it to stain the bacteria negatively. The method has been described earlier.[1]

Similar grids were also prepared from the blood in the gut of mosquitoes fed on control volunteers.

Transmission electron microscopy was also performed with mosquitoes fed artificially on a glucose-water-lepromin mixture. Guts and excreta were mounted on coated grids at regular intervals after feeding and stained with 1% uranyl acetate.

Results

A FLUORESCENT MICROSCOPY: A FEEDING EXPERIMENT ON LEPROMATOUS PATIENTS

Table 1 describes the viability of the ingested *M. leprae* by *A. aegypti* after biting bacilliferous lepromatous patients. The percentage of green solid bacteria on the 1st day was 36%; it increased slightly (48%) on the 3rd day and thereafter it decreased to 26% on the 4th day. After the 4th day no green coloured solid bacteria was seen. The percentage of red coloured solid bacteria increased steadily from the 3rd day onwards. The slight increase of green coloured solid bacteria on the 3rd day might be due to transient bacterial multiplication, which had been confirmed by transmission electron microscopy (Figure 4(d) and (e)). Table 1 further showed that the ingested bacilli quickly became fragmented. Most interestingly the beaded bacilli took red coloured stain.

B LIGHT MICROSCOPY

(1) *Feeding experiment on lepromatous patients*. A total number of 81 prestarved female *A. aegypti* were taken and divided into 2 groups for 2

Table 1. Studies on viability and morphology of *M. leprae* within the gut of the *A. aegypti* after they bit on bacilliferous lepromatous patients

Days of bite on which mosquitoes were sacrificed	Total number of solid green and red coloured bacilli counted	Total number and (%) of green coloured bacilli (viable)	Total number and (%) of red coloured bacilli (non-viable)	Total number of beaded‡ bacilli (red coloured) counted	No. beaded bacilli × 100 / No. solid bacilli
1st	80*	27 (35·75)	53 (64·25)	1	1·25
3rd	46*	22 (47·82)§	24 (52·18)	5	10·87
4th	69*	18 (26·08)	51 (73·91)	27	39·13
5th	8†	0	8‡ (100)	16†	200
6th	36*	0	36‡ (100)	125‡	347·22

* Randomly counted in 3 experiments consisting of 3 mosquitoes.

† Randomly counted in only 1 experiment, consisting of 1 mosquito. The low bacterial count might be due to the fact that this mosquito did not suck blood well.

‡ All ingested *M. leprae* were dead on the 5th day of bite.

§ This rise in the percentage of green coloured bacilli might be due to the bacterial multiplication within 48 h of bite (Figure 4(d) and (e)). Alternatively this initial increase of the percentage of viable bacteria might be also due to a declumping effect as a result of surface changes in the dying mycobacteria.[11]

experiments. In the 1st experiment, 51 mosquitoes were allowed to bite 2 LL patients and, in the 2nd experiment, 30 mosquitoes were fed on 3 LL volunteers.

The blood was found in the gut up to 48 h and thereafter it was absorbed. The incidence of AFB in the gut of mosquitoes was studied 2 h after the bite and thereafter followed daily up to 7 days. Four mosquitoes were sacrified every 24 h, and gut from each insect was dissected separately, smeared and stained for acid-fast bacilli. Thus from the 2 experiments 8 slides were prepared every day. The incidence of positivity was very high initially. Sixty-two per cent of the total number of slides screened showed AFB up to 48 h after feeding (Figure 2(a)). Thereafter not only the incidence of AFB positivity per slide became less but also the bacilli were less in number. However, solidly stained AFB were seen in the gut of mosquitoes up to 120 h following blood meals and thereafter mostly beaded AFB were seen.

(2) *Artificial feeding experiments on glucose-water-lepromin mixture.* In a control experiment, 60 prestarved female adult *A. aegypti* mosquitoes were allowed to feed on the glucose-water-lepromin mixture. Their guts were dissected every 24 h for 5 days and processed for demonstration of AFB by light as well as by electron microscopes.

The number of AFB found in the gut of mosquitoes thus fed artificially were much larger than observed in the gut of mosquitoes fed on leprosy patients. Large globi of AFB, solidly stained, were seen 24 h after feeding. This was probably due

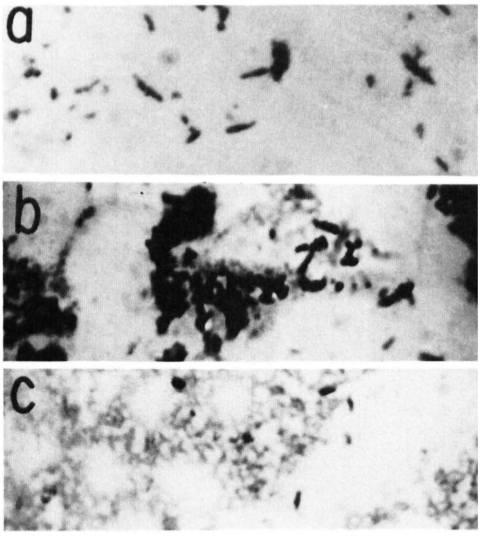

Figure 2. (a) Shows mostly solid acid-fast bacilli and few fragmented *M. leprae* in the gut of one female adult *A. aegypti*, 48 h after biting a lepromatous patient. This is the 2nd feeding experiment in which 30 mosquitoes were allowed to bite 3 lepromatous patients.

(b) Shows large numbers of solidly stained *M. leprae*. These are obtained from the gut of a female *Aedes aegypti* fed on glucose-water-lepromin mixture. The sample was taken 24 h after artificial feeding on lepromin.

(c) Shows fragmented acid-fast bacilli in the excreta of female *Aedes aegypti* 24 h after feeding on glucose-water-lepromin mixture.

to the high concentration of solid bacteria in the lepromin suspension. Unlike the AFB seen in the gut of mosquitoes fed on leprosy patients, practically no fragmented bacilli were seen in this experiment. Even when these mosquitoes were sacrificed 72 h after artificial feeding, the contents of the gut showed globi of solidly stained *M. leprae* and some fragmented bacilli (Figure 2(b)).

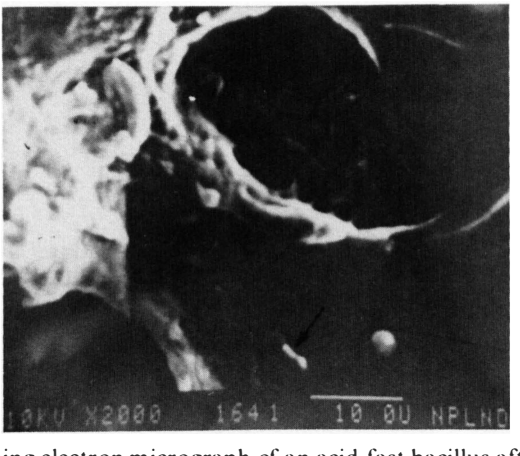

Figure 3. Shows a scanning electron micrograph of an acid-fast bacillus after 3 guts from 3 female *Aedes aegypti* were sonicated. These mosquitoes were allowed to feed on a maculo-anaesthetic leprosy patient who had a bacillary index of +2. Mosquitoes were sacrificed 48 h after biting. The left upper part of the figure shows the collapsed gut membrane. One AFB is seen in dividing stage. ($\times$ 2000).

The excreta collected in this experiment, 24 and 96 h after feeding, however, demonstrated mostly beaded acid-fast bacilli, but the number of bacilli gradually decreased (Figure 2(c)). This experiment was terminated 4 days after artificial feeding.

(3) *Control experiments.* (a) *Control unfed mosquito group.* Twenty control unfed female *A. aegypti* were included in this experiment. None showed any acid-fast bacilli either in the gut or excreta. (b) *Control-fed mosquito group.* Twenty-five control female *A. aegypti* fed on a normal volunteer were followed every 24 h

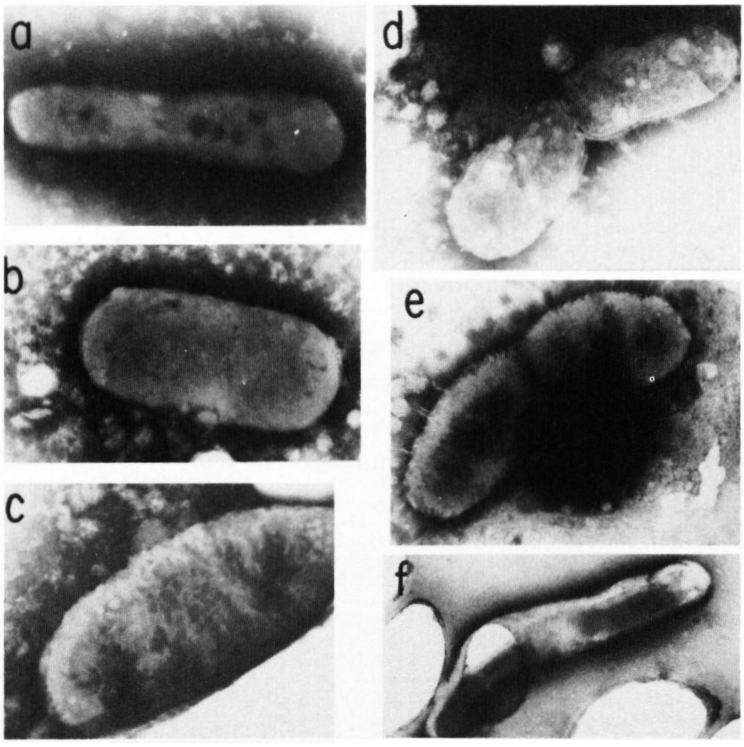

Figure 4. (a) Shows a characteristic ultrastructure of *M. leprae* within the gut of a female *Aedes aegypti* after 24 h of feeding on a maculo-anaesthetic patient. Electron dense areas are visible. (× 55,000).

(b) Shows the ultrastructures of *M. leprae* within the gut of a mosquito 24 h after feeding on a LL patient. Outer membrane of bacteria is not intact. (× 33,000).

(c) Shows the ultrastructures of *M. leprae* in the gut of a mosquito 72 h after biting a LL patient. The particle shows mesosomal structures at the poles. Distinct band structures are visible, which show division of bacteria. (× 42,000).

(d) Shows a dividing mycobacterium within the gut of a mosquito 72 h after feeding on a LL patient. Outer double layered cell membrane is distinctly seen. One of the daughter cells shows budding. (× 42,000).

(e) Shows dividing *M. leprae* in the gut of a mosquito 48–72 h after biting a LL patient. Pilli like structures are visible on both the daughter cells. (× 27,000).

(f) Shows an electronmicrograph of *M. leprae* within the gut of a mosquito artificially fed on lepromin.

for 7 days. No acid-fast bacilli could be demonstrated in the gut or excreta of these mosquitoes.

C ELECTRON MICROSCOPY

Demonstration of the ultrastructures of acid-fast bacilli in the gut of mosquitoes fed on leprosy patients. (i) *Scanning electron microscopy*: Seventy female *A. aegypti* were allowed to bite a maculoanaesthetic patient. Two to three mosquitoes were sacrified every 24 h. Figure 3 illustrates a typical scanning electron micrograph of a dividing acid-fast bacillus within the gut of the mosquito 48 h after feeding. (ii) *Transmission electron microscopy*: Figure 4 (a) illustrates an electron micrograph of an acid-fast bacillus within the gut of a female mosquito fed on a maculo-anaesthetic patient. The picture was taken 24 h after biting. Figure 4(b) and 4(c) show the ultrastructure of *M. leprae* in the gut of the mosquito 24 and 72

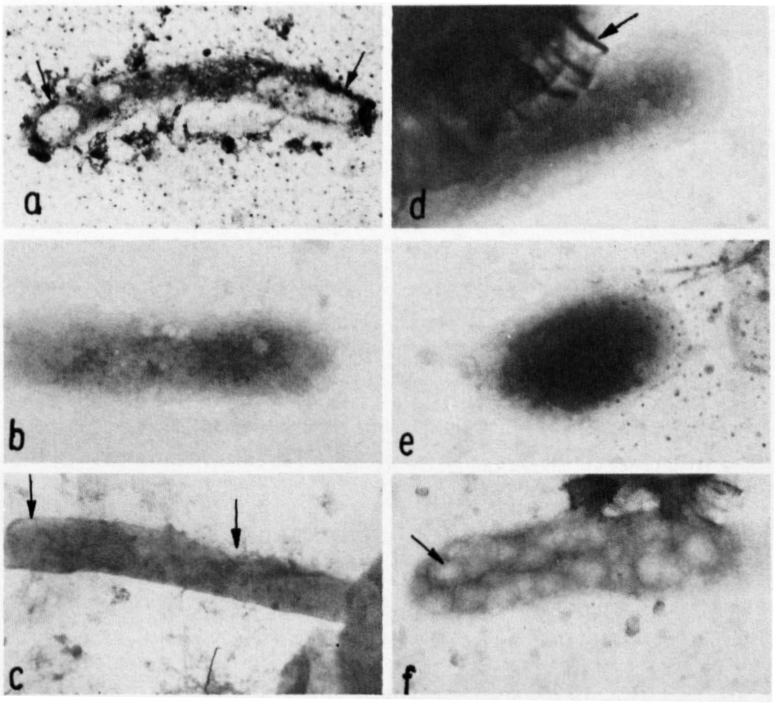

Figure 5. Control electron micrographs of *M. leprae* in lepromin and alterations in the ultrastructures of acid-fast bacilli within the gut of mosquitoes fed on a glucose-water-lepromin mixture.

(a) and (b) Electron micrograph of a pure suspension of *M. leprae* in armadillo-derived lepromin. Mesosomal structures are visible in (a) (× 20,000).

(c) Alteration of the ultrastructures of *M. leprae* within the gut of a mosquito 72 h after feeding on a mixture of glucose-water-lepromin. Double membranes are partially visible. Middle portion of the bacteria is partially digested (× 42,000).

(d), (e) and (f) Alteration of the ultrastructures of *M. leprae* within the gut of a mosquito 96 h after feeding on a mixture of glucose-water-lepromin. Vaculations within the bacteria are seen in (d) and (f). Musculature of the gut of mosquito is also visible in (d) and (f) (× 27,000).

h after biting the LL patient. Figure 4(d) and (e) clearly depict dividing acid-fast bacilli in the gut of mosquitoes after 72 h of biting a LL patient. Figure 4(d) demonstrates a symmetrical membrane structure.

Results of electron microscopy of the guts obtained from mosquitoes fed artificially on the glucose-water-lepromin mixture were similar to that observed in insects fed on leprosy patients (Figure 5). The number of AFB within the gut gradually decreased after artificial feeding. Many AFB were observed up to 96 h after feeding; thereafter occasional AFB were seen. After 4 days of feeding, lysis of AFB started (Figure 5(f)) and by 168 h lysis was almost complete. An electronmicrograph of *M. leprae* in the excreta of mosquitoes fed on the glucose-water-lepromin mixture after 72 h of artificial feeding showed fragmented and disrupted bacteria.

When the guts of mosquitoes fed on healthy volunteers were examined under a transmission electron microscope, digestion of erythrocytes and monocytes was seen after 24 h of biting. Musculature of the mosquito gut as well as virus-like particles was also visible inside the gut.

Discussion

The work of Narayanan *et al.*[9] aroused the interest of leprologists in the possibility of transmission of leprosy by arthropods. They suggested that for successful transmission, 3 essential requirements are necessary: 1, acquisition of sufficient number of living bacilli; 2, sustenance of the acquired bacilli in viable form until they are transmitted in new hosts; and 3, the ability of parasites to multiply in the vector.

To demonstrate the viability of *M. leprae* in the intestines of mosquitoes we have used the fluorescent staining procedure.[7] The staining method incorporated FDA and EB mixture. FDA entered the live cells, enzymatically hydrolysed by acetylesterase, rapidly accumulated in the cytoplasm and appeared green under ultraviolet light indicating viability. EB entered dead cells, combined with DNA and appeared red. Thus viability of the ingested green coloured bacteria was correlated with the presence of native acetylesterase within *M. leprae*. After the 4th day of bite no green coloured bacteria were seen within the mosquito gut indicating thereby the destruction of the enzyme of *M. leprae*.

In order to demonstrate the ability of *M. leprae* to multiply in mosquitoes, arthropods were allowed to bite bacilliferous leprosy patients. In a control experiment, they were also allowed to suck a mixture of glucose-water and armadillo-derived lepromin containing dead *M. leprae*.

The earlier investigators studied the transmission of *M. leprae* by mosquitoes by light microscopy, which had not allowed them to study any alteration in the ultrastructures of *M. leprae* in the adverse environment of the gut of the insects. We therefore decided to study this aspect of transmission of leprosy by mosquito.

As observed by previous workers, we also found that the number of *M. leprae*

in the gut of mosquitoes was larger initially and became scanty in the later part of the week after blood meals as well as after artificial feeding of lepromin. Electron-dense materials (negative staining) have been visible (Figure 4a). Similar electron-dense substances have recently been described by Hirata in longitudinal serial sections of *M. leprae*.[10] Mesosomal structures and nuclear apparatus have also been seen in a dividing bacterium (Figure 4(c)). Most interestingly both transmission (Figure 4(d) and (e)) as well as scanning electron microscopy (Figure 3) and also fluorescent technique could demonstrate bacterial cell division in the gut of mosquitoes within 72 h after biting. Another interesting finding is that solid AFB found in the excreta of the mosquitoes were not only small in number but they were also disrupted and fragmented. No *M. leprae* were found in dividing stage in the gut of mosquitoes which sucked lepromin (Figure 5). The most striking finding is a symmetrical membrane profile (Figure 4(d)). This is characteristic of *M. leprae* and is not shared by other cultivable species of mycobacteria.[8] However it cannot be taken as a definitive identification of *M. leprae*, since during fixation with 1% uranyl acetate, we had not used divalent cations like calcium or magnesium,[8] which might have affected the ultrastructure of *M. leprae*.

We are thus tempted to postulate from the similarity of electron micrographs of the bacteria within the gut of mosquitoes fed on patients (Figure 4) and lepromin-glucose-water mixture (Figure 5) that binary division of *M. leprae* within the gut of *A. aegypti* was possible at least in the early days after blood meals. This view is further substantiated by the observed increase of the percentage of green stained bacilli from 36% to 48% on the 3rd day of bite (Table 1). Since the number of green stained *M. leprae* rapidly decreased in the gut of the mosquitoes after 96 h and since red stained beaded bacilli increased rapidly in number after the 4th day of bite (Table 1) and further since the excreta showed fragmented and disrupted AFB, it is possible that digestive enzymes of the gut of the vectors might have affected the ultrastructures of *M. leprae* and probably have killed the parasites. However, detailed enzyme studies are necessary to substantiate our hypothesis.

Our findings of binary division of *M. leprae* in the gut of mosquito within 72 h after biting leprosy patients (Figure 4(d) and (e)) is contrary to the existing notion that propagation time of *M. leprae* is sufficiently long and the lifespan of the arthropods is relatively short. Thus substantial multiplication of *M. leprae*, if at all, could have not been possible within the gut of the mosquitoes. Therefore the observed transient multiplication of *M. leprae* within the gut of mosquito under an electron microscope might have 2 explanations:

(1) it is possible that under the gut environmental conditions, such as the contaminated water offered to the insects, saprophytic mycobacteria might have multiplied in the gut. But had it been so, the percentage of the green stained AFB should have been progressively increased during all the days of our experiment following blood meals. One might also argue that the staining technique would not differentiate between AFB and any bacterial flora in the gut of mosquito, such

as *E. coli*, so often present in it. We had demonstrated that the intestines of mosquitoes bred under sterile conditions were free from Gram positive and Gram negative bacteria. Furthermore the guts of control unfed mosquitoes and those of mosquitoes fed on normal volunteers showed no AFB like bacilli by EM. Even if the guts of our mosquitoes contained bacteria other than *M. leprae*, then the ratio of the numbers of green to red bacilli should be equal during all periods instead of the observed progressive decrease of green and rapid rise of red bacteria (Table 1). Furthermore the symmetric geometry of the outer and inner membranes of the mycobacteria as observed in the electron micrograph in our study (Figure 4(d)) as well as the similarity of the electron micrographs of *M. leprae* in the gut of lepromin fed insects (Figures 4 and 5) rule out this possibility.[8]

(2) Alternatively binary fission of the *M. leprae* (Figure 4(d)) and 4(e)) which might have started in the circulation of the patient at the time of blood meal was seen within 72 h of the feeding experiment. However, under the adverse environment of the gut of the arthropod, *M. leprae* became quickly non-viable (red stained) and eliminated. Further experiments are necessary to substantiate this posulate.

Acknowledgment

We are grateful to the Indian Council of Medical Research for granting a fellowship to one of the authors (MJ).

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