

Transmission of viable *Mycobacterium leprae* by *Aedes aegypti* from lepromatous leprosy patients to the skin of mice through interrupted feeding

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Summary Female *Aedes aegypti* which took partial blood meals from the skin lesions of untreated lepromatous leprosy (LL) patients were then allowed to continue feeding on 72–96-hr-old Swiss albino suckling mice (Rockefeller strain). The bitten portion of skin was removed, divided into two parts and processed for the extraction of bacilli by two different methods using chloroform and petroleum ether. The proboscis of some of the fed mosquitoes was dissected out and examined for viable bacilli (stained by fluorescein diacetate and ethidium bromide) and acid-fast bacilli (AFB). Out of 50 probosces dissected 45 were found positive for AFB, with bacillary counts ranging up to 246 (average $40.20 \pm \text{SD } 41.80$) per proboscis. The average percentage of viable bacilli (green solid) in the probosces immediately after feeding on LL patients was 43.90 and thereafter it decreased gradually to 3 on the seventh day. In the petroleum ether extract of mouse skin viable bacilli were observed in numbers up to 37 (average $15.25 \pm \text{SD } 10.25$) per smear. The number of fluorescing bacilli (green and red) correlated with the total number of AFB.

Introduction

Little is known about the mode of transmission of leprosy by blood sucking arthropods although leprosy bacilli (*Mycobacterium leprae*) are able to persist for several days in the gut and mouth parts and on the legs of mosquitoes, flies, bed bugs, etc.¹⁻² Under experimental conditions the possibility of transfer of acid-fast bacilli (AFB) from lepromatous leprosy (LL) patients to mouse footpads by *Aedes aegypti* mosquitoes has been suggested by earlier workers.³ Acid-fast bacilli were also demonstrated in the probosces of *Ae. aegypti* up to 156 hr after feeding on LL patients.⁴ However, the viability of the transferred organisms and the survival period of viable bacilli within the mosquito proboscis have not been tested.

The present work quantifies viable *M. leprae* in the probosces of *Ae. aegypti* from 0–9

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days after blood feeding on LL patients and attempts to find out whether, through interrupted feeding, viable *M. leprae* can be transferred mechanically from dermal lesions of LL patients to the skin of mice.

Materials and methods

PATIENTS

All the 25 LL patients were volunteers attending the out-patient clinics of the Calcutta School of Tropical Medicine. Diagnosis was based on clinical examination and microscopic observation of skin smears for AFB. Patients were not given any anti-leprosy treatment before the experiments.

REARING AND FEEDING OF MOSQUITOES

Colonies of *Ae. aegypti* were established in the laboratory under sterile conditions according to the method described by Saha *et al.*⁵ Laboratory reared 5- to 8-day-old *Ae. aegypti* adult females starved overnight were allowed to feed for 40 to 140 seconds on the skin lesions of LL patients (with a Bacillary Index of 3 to 4 on the Dharmendra scale)⁶ using inverted glass tubes with mouths covered with cotton net cloth. Feeding was interrupted before a complete blood meal had been taken and the mosquitoes were placed in the dorsal skin of Swiss albino suckling mice (Rockefeller strain, Laboratory bred, 72- to 96-hr-old reared under sterile conditions) to complete their blood meals 1 hr after the initial feeding.

Twelve suckling mice were used for feeding experiments and each mouse was bitten by 6–10 infected mosquitoes. Similarly for the control experiment the mosquitoes fed on normal subjects were allowed to bite suckling mice.

EXTRACTION OF BACILLI

Two to four slit-skin smears from tissue fluid of dermal lesions of each LL patient were taken on a glass slide, air dried and fixed by single flaming. For extraction of bacilli from suckling mouse skin, the bitten portion of the skin was removed within 2 hr of feeding and divided into two parts. One part was minced, homogenized, and the bacilli were extracted by the chloroform extraction method⁷ and stained for AFB on glass slides. For the other portion of the skin samples petroleum ether was used for extraction of bacilli. Tissue suspensions were prepared by homogenizing 1 part (gram) of tissue in 4 parts (millilitres) of ice cold 0.1 M potassium phosphate buffer, pH 7.2, in a 100 ml stainless steel Sorval Omni Mix tissue homogenizer cup at 15,000 rpm for 2 min on cracked ice. The tissue suspensions were transferred to sterile glass tubes and partitioned by adding 1.0 ml of petroleum ether to 0.1 ml of skin homogenate and mixing for 15–20 sec at room temperature. After 1–2 min to permit phase separation and large pieces of tissue to settle, bacterial smears were prepared from the petroleum ether layer (upper layer) by placing one or two drops of suspension on a microscope slide.

During each experiment the probosces of 4–8 mosquitoes fed on LL patients were individually dissected out under a dissecting microscope in a drop of normal saline on a glass slide within 2 hr of the infective bite. Further samples of mosquitoes were dissected every day for 9 days. Sterile conditions were maintained in all the above preparations throughout the study.

STAINING OF BACILLI

The slides were appropriately stained for AFB by the modified Ziehl-Neelson method and for fluorescing bacilli according to a procedure described by Kvach *et al.*^{8,9} modified suitably as stated below. A mixture of fluorescein diacetate (FDA), 2 µg per ml and ethidium bromide (EB), 4 µg per ml, in 0.1 M potassium phosphate buffer, pH 7.2 was used for fluorescent staining. Two methods were employed for fluorescent staining of mouse skin extracts and human slit-skin smears. Petroleum ether partitioned cells made from infected suckling mouse skin were stained directly on the slides by placing 20–30 µl of FDA/EB working solution then covered with a cover glass and sealed with nail polish to prevent evaporation. The human slit-skin smears made from tissue fluid of dermal lesions of LL patient were stained in a coplin jar containing 45 ml of FDA/EB solutions for 15–20 min and subsequently gently rinsed by two successive washes of phosphate buffer. After air drying, the stained smears were mounted under a cover slip with Eukitt medium. All the slides were incubated at room temperature protected from light for 15–20 min to permit the *M. leprae* cells to stain. The probosces of infected mosquitoes were processed for AFB and fluorescing bacilli staining respectively using the same procedures. Appropriate control experiments were performed simultaneously by feeding the mosquitoes on normal healthy subjects.

AFB stained slides were examined under the oil immersion objective of a Zeiss Axioskop microscope equipped for differential interference contrast with magnification of 10x × 100x. The number of AFB was counted according to the methods described elsewhere in greater detail.^{5,7,10,11,12} FDA/EB stained mycobacteria were observed under the oil immersion objective with incident, ultraviolet illumination equipped with a BP 390–420 exciter filter and red suppression filter. A total of 100–150 fields were examined on each slide. Green-fluorescing bacilli were considered to be viable and red-fluorescing bacilli considered to be dead. Beaded or bipolar green-fluorescing bacilli were considered as viable. Clumps of bacteria were excluded from counting. All the FDA/EB stained slides of probosces, mouse skin extracts or human slit-skin smears were subsequently stained by the modified Ziehl-Neelson method for determination of total AFB. The morphological indices (MI) were calculated for each slide.

Fifty and 35 probosces of infected *Ae. aegypti* were stained for AFB and fluorescing bacilli respectively. For the control experiment twenty and ten probosces of *Ae. aegypti* fed on normal human volunteers were stained for AFB and fluorescing bacilli. Twenty mice skin smears in each group (experimental and control) were examined for AFB and fluorescing bacilli.

Results and discussion

It was noted that both the fluorescent staining methods were satisfactory, green background fluorescence was minimal or absent, and the viable *M. leprae* cells stained rapidly with green fluorescence.

Table 1 shows the number of AFB and fluorescing bacilli in the probosces of *Ae. aegypti* after blood meals on LL patients. Out of 50 probosces dissected AFB were detected in 45 with bacillary counts ranging up to 246 per proboscis. Out of 35 probosces dissected, fluorescing bacilli were detected in 31 with bacillary counts ranging up to 121 per proboscis. Viable bacilli were detected within 2 hr of feeding in all 31 probosces with a

Table 1. Studies on the viability of *M. leprae* in the probosces of *Aedes aegypti* and skin extracts of suckling mice

Source of <i>M. leprae</i>	Total no. of AFB	Total no. of fluorescing (red & green) bacilli	Total no. of green-stained bacilli (viable)	Percentage of green-stained bacilli (viable)	Total no. of red-stained bacilli (non-viable)	Percentage of red-stained bacilli (non-viable)
Proboscis	40.20 ± 41.80 (0-246)	34.40 ± 29.34 (0-121)	17.17 ± 14.40 (0-53)	43.90 ± 20.60 (0-75)	17.20 ± 15.47 (0-68)	41.70 ± 19.90 (0-62)
Skin smear	25.00 ± 16.00 (0-59)	27.05 ± 14.86 (0-57)	15.25 ± 10.25 (0-37)	48.00 ± 20.40 (0-80)	11.80 ± 5.30 (0-21)	42.00 ± 18.80 (0-71)

* All values are given as mean ± SD and range (in parentheses) per proboscis or per skin smear.

bacillary count of green cells ranging up to 53 per proboscis. The average percentage of green solid bacilli immediately after feeding was 43.90, thereafter decreasing gradually to 6% and 3% on the sixth and seventh day after feeding respectively. No green bacilli could be detected in the proboscis on the eighth day. The percentage of red coloured solid bacteria increased steadily from the second day onwards. The survival of *M. leprae* for up to 4 days in the gut of mosquitoes has been reported earlier by Saha *et al.*⁵

Out of 20 smears made from 12 suckling mouse skin extracts and examined for AFB, 17 smears were found positive with a bacillary count of up to 59 per skin smear. In the 20 smears examined for fluorescing bacilli (viable and non-viable) 18 smears were found positive with bacillary count ranging up to 57 per skin smear. Petroleum ether extracts of suckling mouse skin showed up to 80% of green bacilli per skin smear. The remainder of the bacteria stained red (Table 1). The FDA/EB stained slit-skin smears of 20 LL patients showed 35–72% green stained *M. leprae*. The human skin smears from the same subject were simultaneously stained by the Ziehl–Neelson method, further confirmed the total number of bacilli as observed in FDA/EB stained smears.

The *in vitro* methods for the detection of AFB and for the identification and evaluation of *M. leprae* viability were simultaneously undertaken on 20 bacillary extracts obtained from mouse skin and 35 probosces of *Ae. aegypti*. The number of bacteria seen by the two methods showed significant correlation in skin smears ($r = 0.95$, $df = 18$, $P < 0.001$) as well as in probosces ($r = 0.98$, $df = 33$, $P < 0.001$). It is therefore concluded that the two staining methods provide a reasonable measure of the possibility of transfer of *M. leprae* to mouse skin through interrupted feeding by *Ae. aegypti*. Earlier studies have shown that FDA/EB method is a useful assay of bacillary viability with good correlation with the established mouse footpad model.^{9,13,14} Furthermore, neither the proboscis of control unfed mosquitoes nor those of mosquitoes fed on normal human volunteers, nor mouse skin extracts bitten by control mosquitoes showed any AFB by either staining method.

The work of Narayanan *et al.*³ suggested the possibility of mechanical transfer of AFB by arthropods. In their study, multiplication of the non-cultivable AFB, presumed to be *M. leprae* was demonstrated in four instances out of 208 mouse footpads examined after the footpads were bitten by *Ae. aegypti* previously fed on LL patients. Although the positive results obtained were too few to establish any clear conclusion about actual role of blood sucking arthropods in transmission of leprosy but their studies do show the occasional transfer of viable bacilli through interrupted feeding. The low rate of success could have resulted from the propensity of the *M. leprae* to provoke a chronic macrophage inflammation in the mouse footpad, susceptibility of inbred mice and growth kinetics, and immunological response of the mice resulting in failure of bacteria to

proliferate.^{9,11,15,16} On the other hand minimal infective dose of *M. leprae* in mouse has been reported to be as low as 10 bacilli.¹¹ Hence, our results clearly indicate that the viable bacilli may be present in the proboscis of *Ae. aegypti* in sufficient numbers after interrupted feeding on LL patients to initiate multiplication in mouse.

Mechanical transmission of viral disease, for example Rift valley fever, by way of interrupted feeding of mosquitoes has been reported by various investigators.¹⁷ Similarly leprosy is likely to be mechanically transmitted to humans via interrupted feeding of mosquitoes. The transfer of viable AFB from LL patient to mouse skin by *Ae. aegypti* has been clearly demonstrated. Hence epidemiological significance of mechanical transfer of *M. leprae* through blood sucking arthropods should be kept in mind. However, the available findings do not permit us to make a statement on the infective dose for human based on mouse model data. More detailed studies on the actual role of mosquitoes in the transmission of leprosy are now warranted based on prolonged intervention trials of the effect of mosquito control on the leprosy transmission rate in order to prove that insects are a significant vector of leprosy.

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References

- 1 Narayanan E, Shankara Manja K, Kirchheimer WF, Balasubrahmanyam, M. Occurrence of *M. leprae* in arthropods. *Lepr Rev*, 1972; **43**: 194-8.
- 2 Greater JG. The fly as potential vector in the transmission of leprosy. *Lepr Rev*, 1975; **46**: 279-86.
- 3 Narayanan E, Sreevatsa, Kirchheimer WF, Bedi BMS. Transfer of leprosy bacilli from patients to mouse footpads by *Aedes aegypti*. *Lepr India*, 1977; **49**: 181-6.
- 4 Narayanan E, Sreevatsa, Daniel Raj A, Kirchheimer WF, Bedi BMS. Persistence and distribution of *M. leprae* in *Aedes aegypti* and *Culex fatigans* experimentally fed on leprosy patients. *Lepr India*, 1978; **50**: 26-37.
- 5 Saha K, Jain M, Mukherjee MK, Chawla NN, Chaudhury DS, Prakash N. Viability of *Mycobacterium leprae* within the gut of *Ae. aegypti* after they feed on multibacillary lepromatous patients. A study by fluorescent and electron microscope. *Lepr Rev*, 1985; **56**: 279-90.
- 6 Dharmendra. Notes on leprosy: epidemiology of leprosy. Ministry of Health, Government of India, New Delhi, 1960, pp. 174-7.
- 7 Dharmendra, Mukherjee N. A simple method of concentration of leprosy bacilli from 'closed' cases of leprosy. *Lepr India*, 1952; **24**: 169-77.
- 8 Kvach James T, Veras Jose R. A fluorescent staining procedure for determining the viability of Mycobacterial cells. *Int J Lepr*, 1982; **50**: 183-92.
- 9 Kvach James T, Guadalupe M, Strand, Susan H. Staining tissue derived *M. leprae* with fluorescein diacetate and ethidium bromide. *Int J Lepr*, 1984; **52**: 176-84.
- 10 Shepard CC. The nasal excretion of *Mycobacterium leprae* in leprosy. *Int J Lepr*, 1962; **30**: 10-18.
- 11 Shepard CC. *Mycobacterium leprae* in mice; minimal infectious dose, relationship between staining quality and infectivity, and effect of cortisone. *J Bact*, 1965; **89**: 365-72.
- 12 Shepard CC, McRae DH. A method for counting acid-fast bacteria. *Int J Lepr*, 1968; **36**: 78-82.
- 13 Harshan KV, Prasad HK, Chopra NK, Mishra RS, Gogiya P, Nath I. Comparison of radiometric macrophage assay and fluorescein diacetate/ethidium bromide staining for evaluation of *M. leprae* viability. *Int J Lepr*, 1987; **55**: 316-21.

- ¹⁴ Satish M, Rees RJW, Seshadri PS, Nath I. Comparison of radiometric macrophage assay and the mouse foot pad infection for the evaluation of *Mycobacterium leprae* sensitivity/resistance to dapson. *Int J Lepr*, 1985; **53**: 378–84.
- ¹⁵ Shepard CC. The experimental disease that follows the infection of human leprosy bacilli into the foot pads of mice. *J Exp Med*, 1960; **112**: 445–54.
- ¹⁶ Narayanan E, Kirchheimer WF, Bedi BNS. Role of arthropods in the transmission of leprosy: A short review. In *Progress in Immunology of Leprosy*. Talwar GP, Turk JL, Rees RJW (eds), Arnold-Heinemann, 1983; pp. 62–8.
- ¹⁷ Lynn Hoch A, Thomas P., Gargan II, Charles LB. Mechanical transmission of Rift valley fever virus by hematophagous diptera. *Am J Trop Med Hyg*, 1985; **34**: 188–93.

La transmisión de *Mycobacterium leprae* par *Aedes aegypti* des patients de lèpre lépromateuse à la peau des souris à cause de d'une alimentation interrompue

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Sommaire - Des femelles de l'espèce *Aedes aegypti* qui ont pris des repas de sang partiels sur les lésions cutanées des patients de lèpre lépromateuse (LL) non traités, ont continué à s'alimenter sur des souris albinos en étape d'allaitement, de 72 à 96 heures d'âge (race Rockefeller). La portion de peau piqué a été enlevé, coupé en deux parties, et traité pour l'extraction des bacilles par deux méthodes différentes: l'une à base de chloroforme et la deuxième à base d'éther de pétrole. Les trompes de quelques unes des moustiques alimentées ont été découpées et examinés pour la présence des bacilles viables (teintés avec diacétate de fluoréscéine et bromure d'éthidium) et des bacilles acido-résistants (AFB). De 50 trompes examinées, 45 ont donné résultat positif pour AFB, avec comptes bacillaires jusqu'à 246 (compte moyenne de 40,20 ± écart type 41,80) par trompe. Le pourcentage moyen de bacilles viables (solide vert) sur les trompes juste après l'alimentation avec le sang de patients de LL était de 43,90 et à partir de ce point ce chiffre a baissé jusqu'à arriver à 3 au septième jour. Dans l'extrait de peau de souris à base d'éther de pétrole, un nombre maximum de 37 bacilles viables a été observé (compte moyenne de 15,25 ± écart type 10,25) par prélèvement. Le nombre de bacilles fluorescents (verts et rouges) correspondait avec le compte totale de AFB.

La transmisión de *Mycobacterium leprae* viables por *Aedes aegypti* de pacientes con lepra lepromatosa a la piel de ratones por alimentación interrumpida

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Resumen - Hembras de la especie *Aedes aegypti* que habían tomado parte de su alimento de la sangre de lesiones cutáneas de pacientes con lepra lepromatosa (LL) que no habían recibido tratamiento siguieron alimentándose sobre ratones albinos suizos lactantes (cepa Rockefeller) de 72 a 96 horas de edad. Se les quitó la porción de piel que había sido mordida, se dividió en dos partes y fue tratada para la extracción de bacilos utilizando dos métodos distintos: uno a base de cloroformo y otro a base de éter de petróleo. Se disecaron las probóscides de algunos de los mosquitos alimentados y se examinaron para la presencia de bacilos viables (teñidos con diacetato de fluoréscéina y bromuro de etidio) y de bacilos acidoresistentes (AFB). De 50 probóscides desecadas 45 dieron resultado positivo para la presencia de AFB, con recuentos de bacilos llegando hasta los 246 (con media de 40,20 ± desviación estándar de 41,80) por probóscide. El porcentaje medio de bacilos viables (sólido verdoso) en las probóscides inmediatamente después de alimentarse sobre pacientes con LL era de 43,90 y a partir de ahí disminuyó gradualmente hasta llegar a 3 en el séptimo día. En el extracto de éter de petróleo de piel de ratón se detectaron bacilos viables hasta un máximo de 37 (con media de 15,25 ± desviación estándar de 10,25) por frote. El número de bacilos con fluorescentes (verdes y rojos) correspondía al número total de AFB.