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Original Research Article

A New Pathogenicity Model of Leprosy: Mutilation of Toes in Mice Experimentally Infected with *M. leprae*/CAN Bacteria

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Abstract:

Introduction: We describe an animal mutilation model of leprosy, comparable to the human disease, in infant mouse.

Materials & Methods: Infant mouse (6-10 day) inoculated in the mouse footpads (MFP) with 10^7 /10^8 / 10^9 colony forming units (CFU) of *M. leprae* or CAN bacteria adjuvanted with 40 μg sterile collagenase in each MFP; controls consisted of; (i) uninoculated mice of same litters; (ii) mice inoculated with collagenase alone (without *M. leprae* / CAN bacteria); (iii) inoculated with killed bacteria adjuvanted with collagenase, and (iv) those inoculated with *Nocardia brasiliensis* (instead of *M. leprae* / CAN bacteria) with comparable inoculum size.

Results: Earliest suggestive deformities developed around 12 weeks, which manifested clearly and developed progressively giving rise to contractures and mutilations. Studies on these lesions showed significant bacillary proliferation coupled with disintegration / dissolution of the connective tissue and replacement with fibrous tissue. The control uninfected animals did not show such changes, although *N. brasiliensis* could easily multiply in the MFP.

Conclusion: The leprosy bacillus seems to share the capability of producing collagenase and gelatinase, with many members of the Actinomycetes e.g. Nocardiae, nocardioform bacteria, Streptomycetes etc. enabling them to utilise gelatin as a substrate in the human, animal, as well as in the environmental systems, as evidences suggest.

Keywords: CAN bacteria, Collagenase, mutilation, mouse-footpad, clawing, contractures.

Introduction

Despite the fact that human leprosy bacillus is able to multiply in the mouse footpads, armadillos etc., it is unable to produce any mutilation, like that in humans; this could be due to differences in their host susceptibilities and opportunities for developing appropriate pathogenic mechanisms available in different hosts (Dharmendra 1985, Shepard 1985). [1,2]

Our cultivation of the leprosy-derived CAN bacterium (CANb; Chakrabarty et al 1986, 1987) which appears to be a close *in vitro* equivalent of the leprosy bacillus, suggests that collagenase which is possibly an

important virulence factor of the leprosy bacillus, may play a key role in the development of mutilation, by degrading host collagen as a source of gelatin which is a chief metabolite for these bacteria (Chakrabarty et al 1986, 1987, 1991, Dhople & Hanks 1976, Katoch et al 1994). [3-7]

Materials and Methods

Strains

Three strains of CANb (strains LL2, LL9 and R71) were maintained on gelatin minimal (GM) medium with inoculum *Ca.* 10^3 cells, passaged for ≥ 250 generations as pure cultures, to exclude any carry-over of the original leprosy bacillus (Table 1).

Table 1. Inoculum used & their sources.

Strain	Nature of strains	Source
LL2	Human-derived	Isolated & propagated by us in the laboratory
LL9	Human-derived.	Isolated & propagated by us in the laboratory
R71	Armadillo-derived.	RJW Rees, IMMLEP, London.

Media for *in vitro* cultivation

The minimal medium used for the cultivation of the test bacteria comprised (g / 100 ml) KH_2PO_4 0.3; K_2HPO_4 0.1; $(NH_4)_2SO_4$ 0.8, and 0.005% each of $MgSO_4$, $MnSO_4$, $FeSO_4$ and $CuSO_4$, added to 2 g / 100 ml of gelatin and supplemented with 40 μg/ml guanine (Chakrabarty et al 1987); *pH* 7.4-7.6; incubation was at 28^0C, usually for 3-6 weeks. [4]

Mice for experimental infections

Genetically inbred, 'Swiss' white mice, strain A, 6-10 day old, maintained under controlled dietary and hygienic conditions, were inoculated (a batch comprised 20 mice for each CANb strain) into the subcutaneous tissue of both the hind footpads (the front MFPs serving as uninoculated controls) by means of a 26-gauge needle in the head-to-toe direction. Equal number of mice also served as uninfected controls (vide Table 2). For imprint smears and biopsy specimens, the hind limbs of mice (sacrificed by cervical dislocation) were thoroughly washed with Savlon® (ACCI, India), sterile water and rectified spirit, and thereafter dried. The footpads were cut off just above the ankles after snipping away the digits. The bones were separated from the tissues by careful dissection and peeling off of the latter, which were used for further studies.

Table-2. Effects of inoculating MFP with CANb and *M. leprae*

Inoculum	Footpads inoculated (No.)	Effects of inoculating MFP (No.)				
		Permanent contractures	Clawing	Deformities	Mutilations	Total MFP affected
CANb (3 Strains) 10^7-10^9 CFU / MFP **	72	06	14	13	21	54
Leprosy bacillus 10^7, 10^8 or 10^9 CFU / MFP	52	06	06	04	14	30
N. brasiliensis 10^7-10^9 CFU / MFP	14	03	02	02	04	11
Controls:						
Uninoculated cage mates	40	00	00	00	00	00
Litters of infected mice	76	00	00	00	00	00
Collagenase alone	20	00	01	00	00	01
Heated CANb alone	10-	00	00	00	00	00
** +40 μg collagenase /MFP						

Inoculum of CANb / *M. leprae*

Each inoculum comprised 10^7, 10^8 or 10^9 CFU of *M. leprae* / CANb in 0.1 ml of GM liquid medium mixed with 40 μg of collagenase (Type VII, lyophilised, Sigma Laboratories, USA) and was injected into each MFP.

Controls

These included: (1) uninoculated cage-mates of experimentally infected mice, (2) uninoculated forelimbs of inoculated experimental animals, (3) fresh litters born from inoculated (infected) mothers, (4) mice inoculated with sterile collagenase only (without *M. leprae* / CANb), (5) heat killed CANb, and (6) MFP infected with *N. brasiliensis* only.

Histopathology and other studies

Clean, severed away, freshly obtained MFPs were processed using sterile precautions as described above. Tissue juice from such footpads, expressed through a slit, and removed on the tip of sterile scalpels was used to inoculate GM medium with *Ca*. 10^3 bacteria. Imprint smears were prepared by placing small portions of dissected-out footpad tissues, and firmly pressing these between 2 clean glass slides. These were studied by Fite - Faraco stain, using a mixture of equal parts of liquid paraffin and xylol and decolourisation by 1% H_2SO_4 (modified by Wade 1963, Hastings 1985). [8,9] For histopathological studies, tissues were fixed according to Zenker's method (Collee et al 1989) and thin sections (*Ca*. 4 μ) were cut. Smears of bacteria from the footpads, those from the *in vitro* grown cultures, as well as, those from the imprint smears, were stained by the modified Ziehl-Neelsen method (Johne 1885), using a mixture of 1% H_2SO_4 and 90% ethanol. [10,11]

Results

A composite picture of deformities and mutilations observed and recorded is presented in Tables 2 & 3. When the inoculum in the footpads consisted of a mixture of CANb (or *M. leprae*) and collagenase, earliest deformities began to appear around 10-12 weeks after inoculation. Gradually, the deformities progressed to clawing, contractures, loss of toes, and extensive mutilations at a high frequency, after 7-8 months. In animals where inoculum of the *M. leprae* or CANb did *not* contain any collagenase, these could only, very occasionally, produce any clear-cut clawing and a definite mutilation could be seen extremely rarely. Smears from florid mutilation lesions showed extensive infiltration of host tissues by *M. leprae* or CANb, which could be repeatedly isolated from such lesions.

Table 3: Clinical evaluation of footpad pathogenicity in mice.

Inoculum	Macroscopic characteristics of lesions			
(source/culture)	Redness	Swelling	Clawing & Contractures	Mutilation
Uninoculated Control	-	-	-	-
Human LL biopsy derived leprosy bacilli	(+) 1-2* (++) 2-3 (+++) 4-6 (+) 8-10	(+) 2-3* (++) 3-4 (+++) 4-6 (++++) 6-10	(±) 4-8* (+) 8-10 (++) 12-18 (+++) 20-22	(+) 20-22* (++) 22-25 (+++) 25-28 (++++) 28-31
CANb LL2	(+) 1-2 (++) 2-4 (+++) 4-7 (+) 7-10	(+) 2-3 (++) 3-5 (+++) 5-8 (++++) 8-10	(±) 5-8 (+) 8-11 (++) 13-19 (+++) 20-22	(+) 20-22 (++) 22-24 (+++) 24-27 (++++) 27-30

CANb LL9	(+) $^{1-3}$ (++) $^{3-5}$ (+++) $^{5-7}$ (+) $^{7-10}$	(+) $^{3-4}$ (++) $^{4-5}$ (+++) $^{5-7}$ (++++) $^{7-10}$	(±) $^{5-7}$ (+) $^{7-12}$ (++) $^{13-17}$ (+++) $^{17-22}$	(+) $^{17-23}$ (++) $^{23-27}$ (+++) $^{27-30}$ (++++) $^{30-33}$
CANb R71 (Armadillo-derived)	(+) $^{1-2}$ (++) $^{2-4}$ (+++) $^{4-7}$ (+) $^{7-10}$	(+) $^{2-4}$ (++) $^{4-6}$ (+++) $^{6-8}$ (++++) $^{8-10}$	(±) $^{7-11}$ (+) $^{11-15}$ (++) $^{16-19}$ (+++) $^{20-24}$	(+) $^{20-22}$ (++) $^{22-26}$ (+++) $^{27-30}$ (++++) $^{30-32}$
(-) denotes absence of a test parameter; **(+), (++), (+++)** & **(++++),** denotes increasing degree of lesions and **(*)**, on numerals in superscripts, denotes duration of lesions in weeks.				

Discussion

Use of a heavy inoculum of CANb / *M. leprae* together with collagenase, ended up in a loss of toes, and varying degrees of clawing / contractures / deformities at high frequencies, and could be seen progressing on a time-scale, much like the LL cases of human leprosy. This is the first ever regular reproduction of human-type mutilation in an experimental animal.

Our earlier studies (Chakrabarty et al 1991) showed that the CANb or LB which are producers of powerful collagenase themselves are able to degrade tissue collagen substance present in different parts of human / animal systems, leading to a release of gelatin as its degradation product. [5] Thus, endogenously produced gelatin may provide a ready source of growth for the leprosy bacillus (or CANb) both *in vivo* and *in vitro.* The large number of evidences on the leproma juice-derived growth factors (Dhople & Hanks 1976, Dhople & Ibanez 1993) and gelatin as an important metabolic substrate (Katoch et al 1989, Katoch 1994) for the leprosy bacillus (LB) / CANb, thus become explainable. [6,7,12,13] Urea, which is another metabolizable substrate of LB (Chakrabarty et al 1986, 1987), is also present abundantly in the host systems, and may adequately supplement the nutritional requirements of LB / CANb. [3,4] We had also observed earlier that *M. leprae* / CANb can utilise nucleic acid degradation products like purine bases, i.e., by utilising guanine, as well as, xanthin / hypoxanthin. So far, all strains of LB / CANb tested by us suggest that LB is a guanine auxotroph (Chakrabarty et al 1986, Katoch et al 1994) and in absence of preformed, readily available guanine, it can still make do with it by growing at a slower rate, obtaining its requirement of guanine by scavenging the purine bases from dead cells. [3,7] This would also explain that the location and multiplication of the LB selectively within the "lepra" cells or macrophage or Virchow cells; this could be by harvesting guanine molecules from the damaged macrophage nuclei, after these are actively phagocytosed by these macrophages (Chakrabarty et al 1986), in contrast with other host cells, which lack a similar power to phagocytose the LB and provide similar opportunities for intracellular / intranuclear growth. [3]

We carried out tests to detect gelatin in lepromata and blood of LL (20) cases; for this purpose, sera / tissue fluid was diluted from 1:5 through 1:640 in a 2-fold series in saline and mixed with trinitrophenol (dilute) / tannic acid (10% w/v) / chromium dichromate (saturated solution) for development of colour precipitates (British Pharmacopoeia 1980). [14] All active LL cases gave strong (+) reactions at 1:160, while treated LL cases and normal persons showed reactions at $\leq$ 10 dilutions. Standard gelatin solution gave (+) reaction as controls.

In florid cases, a known biochemical process is the extensive destruction of collagen tissues in the body and an excretion of hydroxyproline in the urine (Dharmendra 1985). [1] Although mutilation can frequently occur due to

trauma to, and infection of, the insensitive limbs, possibly without destruction of collagen tissues (Brand & Fritschi 1985), a common underlying pathology in many LL cases seems to be an extension of the lepromatous granulomatous process into the connective tissues of bones and joints, resulting in loss of collagen, leading to their dislocation and sub laxation. [15] This may result in the shortening of fingers / toes by as much as halves and other more severe types of mutilations, even in absence of injury and infection (Dharmendra 1985), as may also occur when the joints become swollen, and the digits angulate and shorten (Pflatzgraff & Bryceson 1985). [1,16] The destruction of collagen tissues in the fingers and toes in such cases possibly accounts for such deformities / mutilations (Dharmendra 1985, Chakrabarty et al 1993, Katoch, Workshop, Orlando 1993). [1,17,18]

Observations of other workers also pointed to a probable role of gelatin in the growth of *M. leprae*, which could be objectively evaluated on the basis of definite increase in the ATP values (Bharadwaj et al 1990, Katoch 1994); it may appear to be a key metabolite, relevant also to *in vitro* growth of *M. leprae* (Workshop, Orlando 1993). [7,18,19] Looking back, the growth factors for leprosy bacillus postulated by earlier workers (Wilson & Miles 1955) and also noted subsequently (Dhople & Ibanez 1993), were most probably gelatin itself, which was employed as a growth factor by Bharadwaj et al 1990, pin-pointed earlier by us (Chakrabarty et al 1987, Chakrabarty et al 1990, Chakrabarty & Dastidar 1993, Chakrabarty et al 1991, Workshop, Orlando 1993) and confirmed later by Katoch (1994). [4,7,12,18-23] Gelatin which is not commonly present in the blood, serum and tissue-fluids of normal individuals appears in the blood etc. of LL patients, possibly being produced endogenously (as suggested by us) by *M. leprae* collagenase, acting on the host tissue collagen (Chakrabarty et al 1993). [23] A state of collagen perturbation had been identified clinically by Dharmendra (1985) which is associated with urinary excretion of hydroxyproline, a breakdown product of gelatin. [1] This, thus explains the collagen-gelatin-amino acid chain sequence as an important biochemical pathology of leprosy, and a metabolic pathway of the leprosy bacillus. [24]

Conclusion

It is known that many members of the *Actinomyces* e.g. nocardiae, nocardioform bacteria, streptomycetes etc. can produce collagenase and gelatinase enabling them to utilise gelatin as a substrate in different environments. The leprosy bacillus seems to share this capability in the human, animal, as well as in the environmental systems, as evidences suggest.

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