Migration and proliferation of Schwann cells in adult human leprous nerve cultures

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Summary The migratory and proliferative activities of Schwann cells affected with leprosy were studied in explant cultures of leprous nerves maintained for 4 weeks *in vitro*. In these cultures, it was observed that Schwann cells harbouring *Mycobacterium leprae* failed to migrate from the explant, attach to the culture surface and proliferate. These cells, therefore, were either sloughed off or still localized to the explant region at the end of the culture period. Hence, no outgrowths of Schwann cells were obtained from highly bacilliferous lepromatous nerve cultures. This was a direct inhibitory effect of the intracellular organism on the host. There was no evidence of the effect being mediated through the release of any soluble product. Unparasitized Schwann cells, however, exhibited normal migration, attachment to culture surface and proliferation. Therefore, a good outgrowth of Schwann cells comparable to that from normal nerve was obtained from tuberculoid nerve cultures. Fewer Schwann cells migrated from the bacteriologically negative lepromatous nerve explant, which displayed a normal proliferative activity. From the borderline tuberculoid nerves, there was migration and proliferation only of unparasitized cells.

This study, thus, demonstrates that *M. leprae* inhibits migratory and proliferative activity of the host Schwann cells.

Introduction

Leprosy is primarily a disease of the peripheral nerve and its Schwann cells.[1,2] In the lepromatous form of the disease, the Schwann cells harbour *Mycobacterium leprae*.[3] While in the tuberculoid form, which shows infiltration by the immunocompetent cells, it does not harbour the causative organism.[1,4] This study was undertaken to observe the alteration, if any, in the functional status of the Schwann cells in these 2 forms of the disease.

Utilizing the *in vitro* nerve culture technique, the migratory and proliferative capacity of the leprosy affected Schwann cells were assessed, as these activities

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precede the interaction and association of Schwann cells with the axons during the process of regeneration and remyelination of the nerve.[5, 6]

Materials and methods

NERVE BIOPSIES

Leprosy patients undergoing routine nerve biopsy for diagnostic purposes were used in this study. A part of the biopsy was collected under sterile conditions in minimal essential medium containing crystalline penicillin (100 IU). Under the dissection microscope, the nerve was cleaned, the perineurium freely incised and the interfunicular connective tissue removed. Each funicle was then cut into 1 to 2 mm squares. Twenty explant cultures were set from each biopsy.

CULTURE TECHNIQUE

The funicular pieces were briefly trypsinized (0·25% in phosphate buffered saline) and explanted on glass coverslips coated with collagen by the method of Bornstein.[7] The coverslips were then transferred to sterile plastic Petri dishes (Falcon, USA), and fed with a growth medium consisting of 75% Dulbecco's modified essential medium, 20% foetal calf serum (Gibco) and 5% chick embryo extract (50% EE), 600 mg% glucose and antibiotics made up of 100 iu of crystalline penicillin, 200 μg streptomycin and 50 iu of mycostatin. Cultures were incubated at 36°C in 100% humidity. On the third day, cytosine arabinoside at a concentration of 10^{-5} M was added to these cultures for 48 h to inhibit the excessive growth of fibroblasts,[8] after which the growth medium was renewed twice a week.

MIGRATION AND ATTACHMENT

Cultures were viewed regularly under the phase contrast optics of an inverted microscope (IM 35 Carl Zeiss). Observations were made on the migration from the explant and the attachment to the culture surface of the outgrowing cells. The drained growth medium was collected and checked for the sloughing of the cells by centrifuging the medium at 800 rev/min. In the event of a cell pellet, smears were prepared on the microscope slides, fixed in 3% formaldehyde for 15 min and stained using Ziehl–Neelsen's method.

PROLIFERATION

The proliferative capacity of the cultured cells was assessed by their ability to synthesize DNA. For this 1 μc/ml of ^{3}H-thymidine (specific activity 15,200 MC/

mmol) was added to the culture for 24 h. Then the coverslips were washed with balanced salt solution, fixed in acid–alcohol and coated with Ilford K5 emulsion diluted 1:1 in distilled water. The emulsion was air dried and the coverslips were stored at 4°C in light-proof boxes. After 7 days, the coverslips were developed, fixed and stained using Ziehl–Neelsen's method.

LIGHT MICROSCOPY

Cultures were fixed for Sudan black staining and light microscopic analysis in 3% glutaraldehyde in 0·1 M sodium phosphate buffer, pH 7·2, overnight at 4°C. They were rinsed, post-fixed in 2% osmium tetroxide in 0·1 M buffer pH 7·2 for 1 h at room temperature, rinsed several times, dehydrated to 70% ethanol and stained with 0·5% Sudan Black in 70% ethanol. Then, the cultures were rehydrated and mounted in glycerine jelly. Replicate cultures were stained for acid-fast organisms by the Ziehl–Neelsen method and for non-specific esterase according to the method described by Bancroft.[9] All the explant cultures were scanned under the light microscope for the type of the cells in the explant mass and in the culture outgrowth.

Results

Nerve biopsies were obtained from 12 patients from the radial cutaneous, sural or ulnar nerves. The patients were classified clinically, bacteriologically and histologically as tuberculoid, borderline and lepromatous according to the Ridley–Jopling classification.[10] Two normal nerve biopsies were taken from volunteers. Relevant clinical data of the biopsied nerves is presented in Table 1.

In both the normal and the leprous nerve explant cultures, 2 types of cells were identifiable, the Schwann cells and fibroblasts. The Schwann cells were identified under phase-contrast optics by their long spindle shape, denser cytoplasm and narrow elongated nucleus. These cells were positively stained with Sudan Black and non-specific esterase and exhibited slow proliferation. Fibroblasts were recognized by their broad flattened morphology, weak staining with Sudan Black and non-specific esterase and formation of underlying carpet layer to Schwann cells (Figure 1). The proportion of these 2 cell types varied depending upon the type of leprous nerve explant culture. The proportion of Schwann cells in the culture outgrowth of different types of leprous nerve explant cultures is depicted in Table 2 and their proliferative activity is compared in Table 3.

TUBERCULOID NERVE

Within 3–4 days of culture, the migration of cells from the explant became evident. The light microscopic picture of the outgrowth from a tuberculoid nerve

Table 1. Clinical details of patients from whom nerves were biopsied

Patient No.	Age	Sex	Diagnosis	Duration of leprosy (years)	Treatment (years)	Nerve
FMR 36/82	17	F	Normal	—	—	Digital nerve
FMR 15/83	13	M	Normal	—	—	Sural
FMR 11/83	18	F	Lepromatous +ve*	2	Untreated	Index branch radial cutaneous
FMR 2/83	25	F	Lepromatous +ve	12	2 months	Index branch radial cutaneous
FMR 20/83	35	F	Lepromatous +ve	7 months	Nil	Ulnar (dorsal) branch
FMR 12/81	35	M	Lepromatous −ve*	12	12	Ulnar
FMR 44/82	—	M	Lepromatous −ve	32	32	Index branch radial cutaneous
FMR 25/82	14	M	Borderline tuberculoid	1	1	Ulnar
FMR 30/82	19	M	Borderline tuberculoid	3	2	Index branch radial
FMR 4/83	14	M	Tuberculoid	1	6 months	Cutaneous branch ulnar
FMR 21/82	34	M	Tuberculoid	5	4	Radial cutaneous
FMR 16/83	21	M	Tuberculoid	1	1	Ulnar

* Bacteriological status of the skin smear

Table 2. Outgrowth characteristics of leprous nerve explant cultures. Comparisons made between 20-day-old cultures

Nerve	Explant		Proportion of cell types in the outgrowth*	
	Number of cultures	Cellular outgrowth (%)	Schwann cells (%)	Fibroblasts (%)
Normal	40	85·0	89·3 ± 6·4	10·7 ± 2·1
Lepromatous −ve	40	75·0	21·3 ± 4·8	78·5 ± 10·2
Lepromatous +ve	160	0·0	—	—
Borderline tuberculoid†	60	80·0	53·1 ± 8·2	46·9 ± 9·4
Tuberculoid	120	87·5	82·0 ± 10·2	18·0 ± 3·2

* Results represent mean ± standard deviation. Cells were counted from several fields of each culture under × 63 oil immersion objective of standard WL microscope (Carl Zeiss).

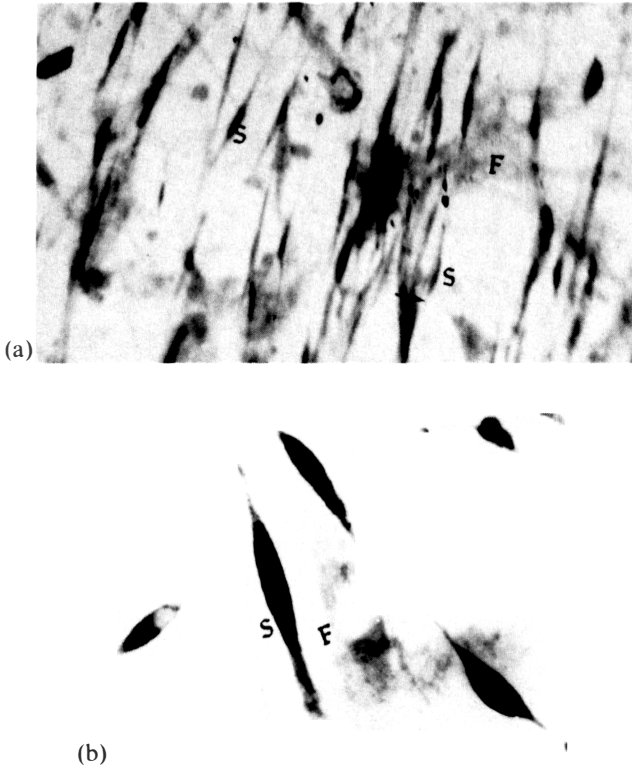† Outgrowth only of cells not harbouring bacilli.

(a)

(b)

Figure 1. Relationship of Schwann cells and fibroblasts in tuberculoid nerve explant culture. Schwann cells (S) lying over the fibroblasts (F): (a) Ziehl–Neelsen stain (× 1025); (b) Non-specific esterase (× 1200).

Table 3. Proportion of Schwann cells cultured from leprous nerves incorporated with ^{3}H-thymidine

Nerve	Schwann cells labelled (%)
Normal	$49 \cdot 2 \pm 5 \cdot 3$
Lepromatous −ve	$33 \cdot 1 \pm 4 \cdot 2$
Lepromatous +ve	—
Borderline tuberculoid	$40 \cdot 0 \pm 3 \cdot 2$
Tuberculoid	$48 \cdot 2 \pm 6 \cdot 4$

Results represent mean ± standard deviation of 10 cultures. Cells were counted from several fields of each culture under × 63 oil immersion objective.
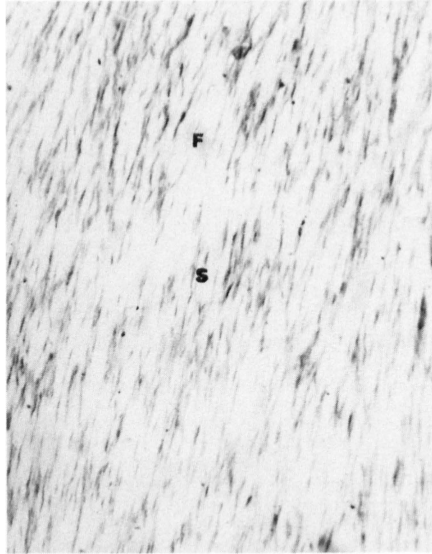
Figure 2. A representative light microscopic picture of a portion of tuberculoid nerve explant culture. Note large number of Schwann cells and a few fibroblasts. Sudan Black B stain (× 350).

Figure 3. A portion of autoradiogram of 21-day-old tuberculoid nerve explant culture. Schwann cell nuclei are heavily labelled with ^{3}H-thymidine (× 1200).

explant is shown in Figure 2. The culture outgrowth is dense and is made primarily of Schwann cells. On quantitation, the Schwann cells formed 82%, while fibroblasts formed 18% of the outgrowth (Table 2). The rich outgrowth of Schwann cells in these cultures was due both to the good migration from explant as well as active proliferation of the Schwann cells *in vitro*. At the end of 3 weeks in culture, 48·2% of the Schwann cells were synthesizing DNA (Figure 3 and Table
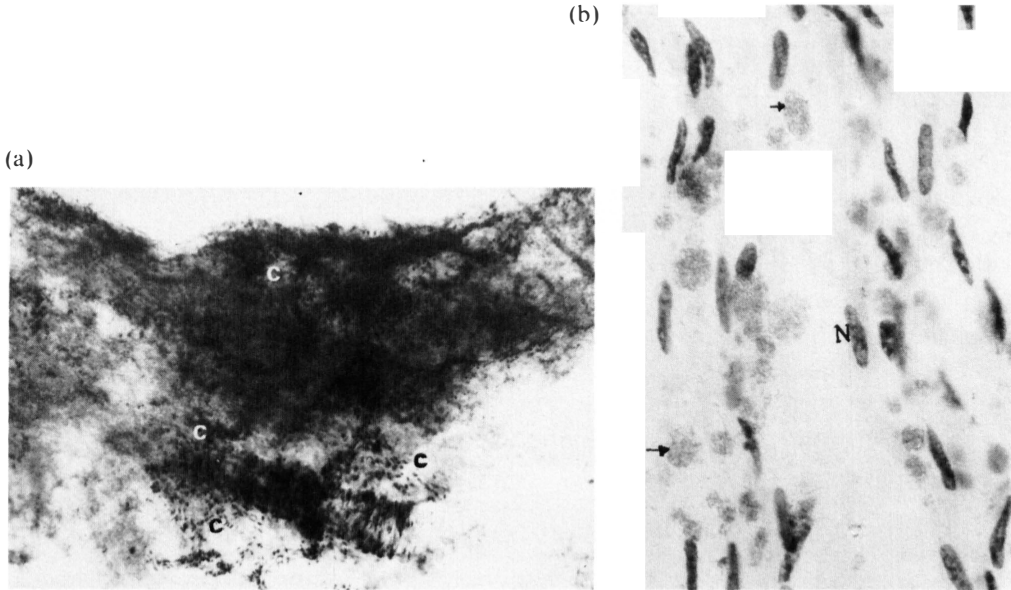
(b)

(a)

Figure 4(a). Bacteriologically positive lepromatous nerve explant containing cells (C). No evidence of cellular outgrowth from explant even after 3 weeks of incubation *in vitro* (× 52).

Figure 4(b). Autoradiogram of teased nerve fibres of bacteriologically positive lepromatous nerve maintained for 7 days *in vitro*. Note the intracellular bacilli (arrow) and non-incorporation of ^{3}H-thymidine by the cells. N = Nucleus (× 850).

3). The cellular outgrowth from the tuberculoid nerve explant was comparable to that from the normal human nerve explants with respect to density and proportion of cell types (Table 2).

BORDERLINE TUBERCULOID NERVE

Outgrowth from the borderline tuberculoid explants contained predominantly Schwann cells not harbouring *M. leprae* even though the explant region contained a mixed population of cells, both *M. leprae* harbouring and non-harbouring. Schwann cells formed 57% of the cellular outgrowth. These cells showed proliferative activity (Table 3).

BACTERIOLOGICALLY POSITIVE LEPROMATOUS NERVE

There was no migration of cells from the explant of highly bacilliferous nerve and hence no outgrowth of cells from these nerves, although the explant region contained viable looking cells loaded with *M. leprae* (Figure 4(a) and (b)). Some of these nerves were teased and maintained *in vitro*. The Schwann cells contained bacilli in clumps and globi, and did not incorporate ^{3}H-thymidine. Therefore in

these cultures there was no activity of migration, attachment and proliferation of the cells throughout the culture period. From time to time there was sloughing of the cells, which when smeared and stained using Ziehl–Neelsen's method, revealed cells to be loaded with acid-fast organisms. At times, there was evidence of attempted extension or migration of cells from the edge of the explant, which when followed, were found to be floating in the medium suggesting their inability to attach to the culture surface.

The lack of migratory and proliferative activity of *M. leprae*-harbouring Schwann cells was a direct effect of the organism on the host cells as the supernatant from the bacteriologically positive lepromatous nerve cultures had no effect on the outgrowth of cells from normal and bacteriologically negative lepromatous explant cultures, thus ruling out the possibility of the effect being mediated through any soluble product.

BACTERIOLOGICALLY NEGATIVE LEPROMATOUS NERVE

In these cultures, there was predominant outgrowth of fibroblasts, Schwann cells forming only 21·5% of the cellular outgrowth, a value much lower compared to that of normal or tuberculoid explant cultures. The low proportion of Schwann cells in these cultures was due to migration of fewer cells from the explant. A good proportion of migrated cells synthesized DNA (Table 3).

Discussion

The nerves of leprosy patients were cultured *in vitro* in order to study the migratory and proliferative function of Schwann cells in this disease. The results demonstrate that intracellular *M. leprae* inhibit the migratory and proliferative functions of the host Schwann cells without affecting the neighbouring unparasitized Schwann cells. Cells not harbouring bacilli exhibited normal migration, attachment to the culture surface and proliferation. In these cultures Schwann cells were identified by their characteristic bipolar spindle shape, a criterion also utilized by others.[11–13] The light microscopic picture of these leprous Schwann cells cultured in this manner were identical to that described in another study[11] in cultures of normal nerves. These observations demonstrate that the light microscopic morphology of unparasitized Schwann cells is not altered in this disease. These cells also stain with Sudan Black B and non-specific esterase more intensely than the fibroblast. The specific staining effect of Sudan Black B and non-specific esterase has also been reported.[12]

The proportion of Schwann cells in the outgrowth of the leprous nerve cultures was determined by the proportion of *M. leprae* not harbouring Schwann cells in the explant which in turn was determined by the types of leprosy the nerve was affected by. Therefore, maximum outgrowth of Schwann cells was observed

in the tuberculoid nerve explant culture. However, these results do not suggest that the Schwann cells are not affected in the tuberculoid form of the disease, but demonstrate that a large proportion of the Schwann cells present in the nerve in this form of the disease have normal proliferative and migratory activity.

The inhibitory effect of *M. leprae* on the proliferative and migratory activity of Schwann cells observed in this study supports our earlier observations on organized nerve culture in which we reported that the intracellular *M. leprae* inhibited the proliferative activity, as well as the alignment and association with the axons of host Schwann cells.[14, 15] The mechanism by which *M. leprae* alters the functional status of the parasitized host cell is not yet understood and is under study. These organisms are not toxic and the protein synthesis of the host is unaffected.[15] But physical occupation of the host cytoplasm by these organisms may alter the organization as well as the content of the cytoskeletal element, which determines the proliferative as well as the migratory activity of the cells in general.[16, 17]

Since there is extensive collagenization of the nerve in chronic lepromatous leprosy,[18] it could be postulated that the cells harbouring *M. leprae* could not migrate out of the explant because of entrapment by the collagen matrix. That this is not so was shown by the ability of the cells from the equally highly collagenized bacteriologically negative nerves[19] to migrate out of the explant. Further, in borderline tuberculoid leprosy where there was a fairly even distribution of cells containing *M. leprae* and those not containing these organisms, the ability of only the cells not containing *M. leprae* to migrate also confirms that this is a defect induced in the host cells by *M. leprae*.

The properties of migration, attachment and proliferation of Schwann cells in the peripheral nerve are important for the association and interaction with axons as well as for the organization of the nerve during development.[20, 21] In the adult this process operates during the regeneration of the nerve.[22, 23] Our observations indicate that the cells harbouring *M. leprae* may not be able to participate in such regenerative processes of the nerve. However, with prolonged chemotherapy, if the intracellular bacilli are cleared, these properties may be restored as observed in the bacteriologically negative lepromatous nerve explant cultures. Poor regeneration seen in the tuberculoid spectrum of the disease may be due to the degeneration of the axons as well, possibly induced by the infiltrating granuloma,[24, 25] since these Schwann cells *in vitro* display normal proliferation and migration.

This study also demonstrates that it is possible to cultivate Schwann cells *in vitro* from the leprous nerve, thus providing possibilities for the study of the cell at the membrane and molecular level in this disease.

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