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A Comparison of the Expression of NGFr, PGP 9.5 and NSE in Cutaneous Lesions of Patients with Early Leprosy Using Immunohistochemistry[1]

Sergio Luiz Gomes Antunes, Euzenir Nunes Sarno, Gunilla Holmkvist, and Olle Johansson[2]

Peripheral neuropathy is the chief cause of the physical deformities and disabilities found in leprosy ([3]). The peripheral nerves in leprosy are affected by either an epithelioid- or a *Mycobacterium leprae*-glutted histiocytic infiltrate accompanied by a reduction in the number of nerve fibers ([10–14]). In leprosy, the early pathogenic mechanisms of the nerve lesion have not yet been clarified. Although immunopathogenic processes are likely to be involved in causing leprosy nerve damage ([21]), Jacobs, *et al.* ([9]) and Shetty, *et al.* ([18]) do not rule out the existence of a direct pathogenic mechanism in leprosy neuropathy.

Neuron-specific enolase (NSE) and the protein gene product 9.5 (PGP 9.5) are widely utilized as neuronal markers in studies on peripheral nerve pathologies ([11–12]). These studies have shown the extent of peripheral nerve damage in target organs in diseases in which the peripheral nervous system was involved.

The NGF family of neurotrophins binds to the nerve growth factor receptor (NGFr) ([13]). It seems that the study of NGFr expression might be a good way to approach the investigation of the functions of the peripheral nervous system ([6]). To date, neurotrophism in leprosy has only been studied by Anand, *et al.* ([1]), who detected depletion of NGF in the skin of leprosy patients by an enzyme-linked immunoassay (ELISA). Attempts to detect the existence of neurotrophic disturbances by examining the immunohistochemical expression of NGFr were carried out in peripheral neuropathies other than leprosy ([17, 19]) and in amyotrophic lateral sclerosis ([2]).

The search for an early pathogenesis of leprosy neuropathy is warranted by the need to prevent the severe disabilities present in the advanced stages of the disease. The immunohistochemical expression of the neuronal proteins PGP 9.5 and NSE, as well as the the low-affinity NGFr expression, as a preliminary approach to neurotrophism in leprosy were investigated in order to detect early alterations of neurons and the relationship of these changes to the initial restricted leprosy infiltrate.

According to Gupte ([8]) early leprosy is characterized by the presence of at least two of the following signs: macular lesions, sensory disturbances, nerve thickening and positivity for acid-fast bacilli (AFB). We were also interested in the NGFr expression as a preliminary approach to neurotrophism in leprosy.

MATERIALS AND METHODS

Eleven biopsies of 11 untreated early leprosy patients (Table 1) and samples of corresponding normal skin areas from 7 plastic surgery patients and 2 volunteers, for utilization as an age- and sex-matched control group, were selected for this study.

The sensory function of the skin was evaluated according to the responses of the patients to different sensory stimuli. Tubes filled with warmed or cold water, a swab of

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[2] S. L. G. Antunes, M.D., Experimental Dermatology Unit, Department of Neuroscience, Karolinska Institute, Stockholm, Sweden and Laboratory of Leprosy, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. E. N. Sarno, M.D., Laboratory of Leprosy, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. G. Holmkvist, Technician; O. Johansson, Ph.D., Experimental Dermatology Unit, Department of Neuroscience, Karolinska Institute, 171 77 Stockholm, Sweden.

Reprint requests to Dr. Johansson.

TABLE 1. *Clinical data of patients.*

	Age (yrs.)	Sex	No. lesions	Lesion sites	BI[a]	Sensory loss sites	Paresthesia sites	Nerve enlargement	Nerve tenderness	Infiltrate type	AFB in biopsy specimen	Diagnosis[e]
1	51	M	3	Arm, forearm[b]	0.33	Lesion	—	—	—	EG[c]	+	BT
2	30	F	1	Shin	0.16	Lesion	—	—	—	EG	—	BT
3	33	M	1	Arm	0	Arm, lesion	—	Auricular	Ulnar	EG	—	BT
4	20	M	1	Forearm	0	Arm, lesion	Elbow	Ulnar	Ulnar	EG	—	BT
5	48	F	4	Knee, arm[b], forearm	0.33	Arm, lesion	—	—	—	EG	—	BT
6	33	F	1	Knee	0	Lesion	Hand	—	—	EG	—	BT
7	13	M	2	Thigh	0	Lesion	Knee	Auricular, ulnar	Ulnar	MCI[d]	—	IL
8	21	M	1	Cheek	0	Lesion	—	—	—	MCI	—	IL
9	49	M	1	Knee	0	Lesion	—	Auricular, ulnar, posterior tibial, fibular	—	EG	—	BT
10	24	M	4	Thigh, forearm,[b] back	0	Lesions	—	Ulnar	—	MCI	+	IL
11	21	F	1	Thigh	0	Lesion	—	—	—	MCI	—	IL

[a] BI = Bacterial index.
[b] Biopsy site.
[c] EG = Epithelioid granuloma.
[d] MCI = Mononuclear cell infiltrate.
[e] BT = Borderline tuberculoid leprosy; IL = indeterminate leprosy.

cotton, and a pin were utilized as stimuli for testing thermal, touch and pain sensitivity, respectively. All of the patients showed abnormal responses to these tests. Histamine was injected into the lesion to test the functional integrity of nerve terminals. Normally, histamine induces the formation of a reddish papule and an erythematous halo at the site of injection. However, none of these patients showed these expected responses. Three individuals had AFB-positive skin smears, but AFB were found only in the biopsy specimens of two patients.

Histopathological findings [hematoxylin and eosin (H&E) and Wade stains] showed a mononuclear inflammatory infiltrate adjacent to the adnexial structures and to the nerve branches of the dermis. In addition to the perineural mononuclear cell infiltrate (MCI), seven patients had a single, immature epithelioid granuloma in the dermis close to the vascular structures.

All patients who had a negative AFB test were suspected to have the disease, taking into account the clinical manifestation, the abnormal response to the histamine test, and the presence of perineural and periadnexial inflammatory infiltrate in the histopathological examination. At the end of multidrug therapy, the diagnosis of leprosy was felt to be confirmed in all of the patients based on the total remission of all pre-existent lesions and neural symptoms.

The skin fragments were fixed in a saturated solution of picric acid in 10% formalin for 2 hr at 4°C and rinsed in phosphate buffered saline (PBS) to which 10% sucrose was added. They were snap-frozen in CO_2, cut as 14-μm-thick sections, using a Microm cryostat (Heidelberg, Germany), and thawed onto glass slides.

An indirect immunofluorescence investigation ([4]) was performed using rabbit anti-human PGP 9.5 (1:2000; UltraClone, Cambridge, U.K.), rabbit anti-human NSE (1:800; UltraClone) and mouse anti-human low-affinity NGFr (1:100, Amersham Sweden AB, Solna, Sweden) as primary antibodies. The secondary antibodies used were rhodamine-conjugated goat anti-rabbit immunoglobulin (1:80; Boehringer, Mannheim, Germany) and rhodamine-conjugated goat anti-mouse immunoglobulin (1:80; Boehringer), respectively. The slides were mounted on buffered glycerine, with p-phenylenediamine (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) anti-fading ([15]), and were observed in a Nikon photomicroscope (Microphot-FXA). Control experiments were performed by using blocked primary antibodies. The secondary antibodies were controlled by omitting the primary ones.

The amounts of immunoreactive nerve fibers were determined according to the semiquantitative numerical estimates in the skin of both groups. The numbers of positive fibers were shown as follows: few (+), average (++), and many (+++). The sections were examined by two investigators.

RESULT

Significant reductions in the amounts of PGP 9.5- and NSE-immunoreactive nerve fibers were found in all regions of the leprosy biopsies, which did not occur in the correspondent regions of the normal specimens (Figs. 1a, 1b; 2a, 2b; Tables 2 and 3). This reduction was also seen in the epidermis in the regions adjacent to adnexial structures and in the interstitium of arrectores pili muscles. However, both control and pathological groups displayed the same NGFr-positivity pattern (Figs. 3a, 3b; Table 4). Moreover, immediately adjacent sections of the same leprosy specimen were NGFr-positive (Fig. 4a), NSE-negative (Fig. 4b), and PGP 9.5-negative. Furthermore, NGFr-positive basal epidermal cells (Figs. 3a, 3b), were observed in both groups, but with a somewhat reduced staining intensity in the leprosy group.

DISCUSSION

Extensive dermal regions with reduced numbers of PGP 9.5- and NSE-immunoreactive nerve fibers were only sparsely occupied by a few inflammatory cells encircling adnexial structures and nerve branches (the inflammatory infiltrate occupied much less than one tenth of the biopsy sections). Selective alterations in the expression of neuronal proteins, unrelated to the regionally restricted and incipient early leprosy infiltrate were found in this study. In addition to corroborating Karanth, *et al.*'s findings ([12]) in regard to the negativity of these protein expressions, PGP 9.5- and NSE-negativity in the early leprosy lesions do not necessarily indicate absence of nerve fibers, as

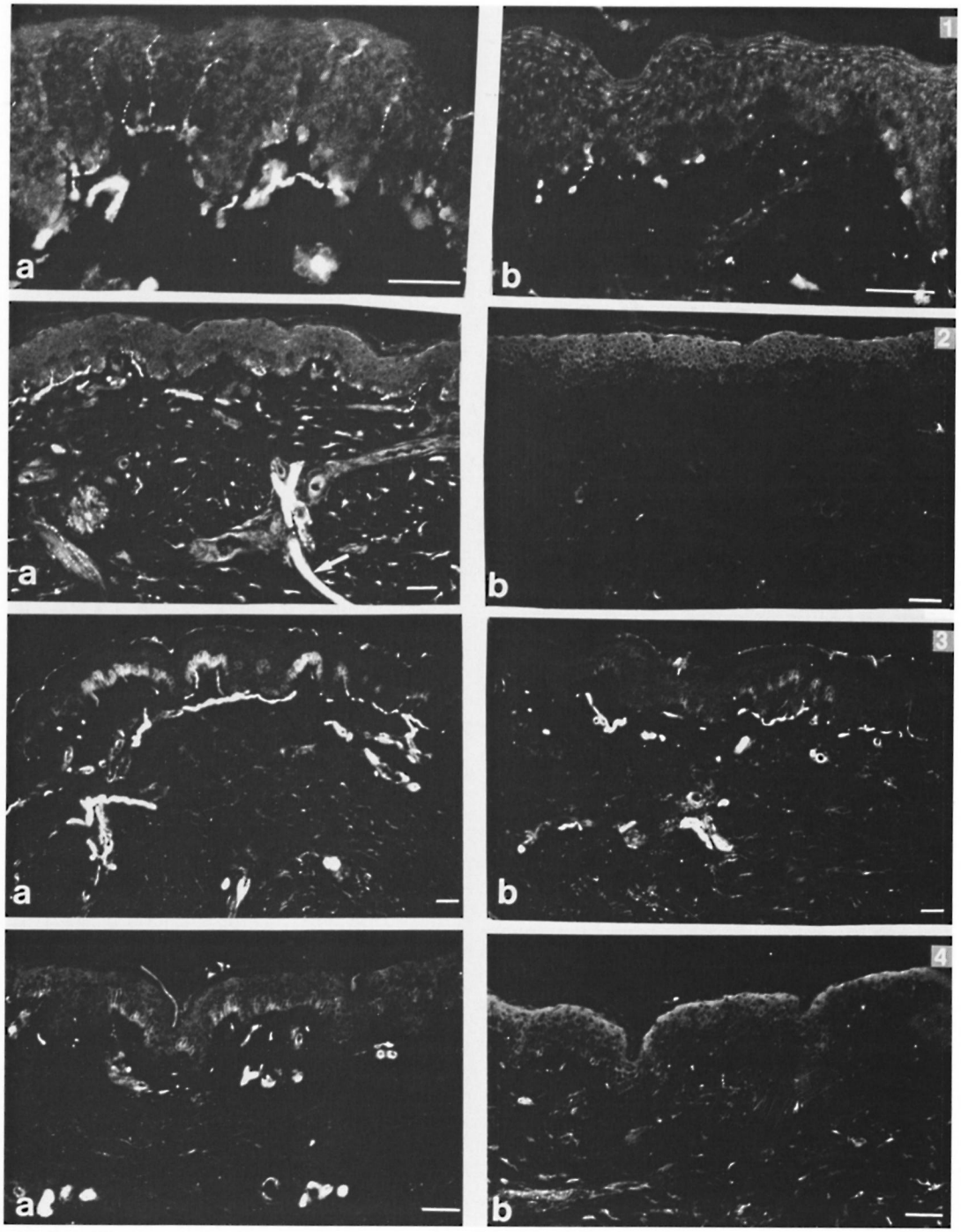

Fig. 1. A greater number of PGP 9.5-immunoreactive nerve fibers in the control (**a**, +++) than in the leprosy skin (**b**, 0) (indirect immunofluorescence; scale bar = 50 μm).

Fig. 2. A greater number of NSE-immunoreactive nerve fibers in the control (**a**, ++) than in the leprosy skin (**b**, 0). A long and thick segment of a cutaneous nerve is also seen in (**a**) (arrow) (indirect immunofluorescence; scale bar = 50 μm).

Fig. 3. Equivalent patterns of NGFr-immunoreactivity in the control (**a**, +++) and in the leprosy skin fragment (**b**, +++). There is a reduced staining intensity of basal epidermal cells in the leprosy specimen (indirect immunofluorescence; scale bar = 50 μm).

TABLE 2. *Reduction of PGP 9.5-immunoreactivity in the cutaneous lesions of leprosy patients (L) compared to the PGP 9.5-positivity in the body region-, age-, and sex-matched specimens of the control group (C); a semi-quantitative numerical estimate.*[a]

	epi		subepi		sg		dv		hf		apm	
	C	L	C	L	C	L	C	L	C	L	C	L
1	+	0	++	0	++	0	++	0	++	0	+++	0
2	++	0	+++	0	++	++	++	++	ND		+++	++
3	0	0	++	+	++	+	ND		ND		++	+
4	++	0	+++	+	ND		+	0	+	0	++	+
5	++	0	++	0	++	0	ND		+++	0	+++	+
6	+++	0	+++	0	+++	0	+++	0	ND		ND	
7	++	++	++	++	++	++	+	+	ND		+	0
8	+	0	+	0	ND		ND		++	+	++	0
9	+	0	++	0	++	0	+	0	ND		++	0
10	++	0	+++	++	+++	++	+++	++	+++	+	+++	+
11	+	0	++	+	++	+	+	+	ND		+	+
T	17	2	24	7	20	8	14	6	11	2	22	7

[a] Abbreviations of the histological cutaneous regions: epi = in the epidermis; subepi = in the subepidermal region; sg = adjacent to sweat glands; dv = adjacent to dermal vessels; hf = adjacent to hair follicle; apm = in the arrectores pili muscle. ND = structures of this type were not found in any of the sections among either control or leprosy specimens, so that comparisons were Not Done; T = sum of the scores of each cutaneous region.

demonstrated by the NGFr-positive expression coupled with PGP 9.5- and NSE-negativity in the immediately adjacent sections of the leprosy specimen.

The lack of a topographical relationship between the extensive dermal regions devoid of immunoreactive fibers and the restriction of the inflammatory infiltrates to adnexial structures and nerve branches suggests the existence of a pathogenic mechanism in the early stages of leprosy neuropathy which may act relatively independent of the specific inflammatory process. Pathogenetic mechanisms of leprosy neuropathy other than the immunoinflammatory response occurring in the nerve have been suspected by Jacobs, *et al.* ([9]) and Shetty, *et al.* ([18]). These authors have reported axonal atrophy, subperineurial edema, and paranodal demyelination in the peripheral nerves of leprosy patients. Axonal atrophy and subperineurial edema were detected through ultrastructural examination of nerves which had a normal appearance by light microscopy. However, these findings have not yet been completely explained ([9]). The paranodal demyelination was detected through the examination of teased axons from pe-

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Fig. 4. NGFr positivity (**a**, ++) and NSE negativity (**b**, 0) in immediately adjacent sections of a leprosy specimen. The weaker positive signals in **b** are not specific and represent auto-fluorescent dermal elastic fibers (indirect immunofluorescence; scale bar = 50 μm).

TABLE 3. *Reduction of NSE-immunoreactivity in the cutaneous lesions of leprosy patients (L) compared to the NSE-positivity in the body region-, age- and sex-matched specimens of the control group (C); a semi-quantitative numerical estimate.*[a]

	epi		subepi		sg		dv		hf		apm	
	C	L	C	L	C	L	C	L	C	L	C	L
1	+	0	+++	0	+++	+	++	0	ND		++	0
2	+	0	++	0	+++	++	ND		+++	+	ND	
3	+	0	++	+	++	+	+	0	+	++	++	+
4	+	+	++	+	ND		+	++	ND		++	+
5	+	0	++	0	ND		++	0	++	+	+++	+
6	++	0	++	0	++	0	++	0	++	0	ND	
7	++	++	++	++	++	+	++	++	ND		0	++
8	+	0	+	0	+++	0	+	+	++	0	++	+
9	0	0	+	0	+	0	+	0	ND		++	0
10	+	+	++	++	+++	++	++	+	ND		+++	+
11	+	0	+	+	++	+	0	0	ND		ND	
T	12	4	20	7	21	8	14	6	10	4	16	7

[a] Abbreviations of the histological cutaneous regions: epi = in the epidermis; subepi = in the subepidermal region; sg = adjacent to sweat glands; dv = adjacent to dermal vessels; hf = adjacent to hair follicle; apm = in the arrectores pili muscle. ND = structures of this type were not found in any of the sections among either the control or leprosy specimens, so that comparisons were Not Done; T = sum of the scores of each cutaneous region.

ripheral nerves ([9]), and was not considered to result from an auto-immune response. Hypothetically, the axonal atrophy reported by Shetty, *et al.* ([18]) may reflect a decrease in the expression of neuronal enzymes, such as PGP 9.5 and NSE, in addition to other proteins. The role of the immunoinflammatory mechanisms in causing late nerve destruction in leprosy cannot be disregarded; however, it is important to identify the critical factor that induces mononuclear cell migration into the nerve. This critical event may arise either from altered nerves or from the bacterial etiologic agent. The blood-nerve barrier must be damaged in the early stages of leprosy to allow for infiltration by leukocytes. These cells are not found in a normal endoneurial compartment ([19]). The events occurring upon the arrival of bacteria to the nerves up to the initiation of the specific immunoinflammatory response require further investigation.

M. leprae itself could cause the early biochemical and molecular alterations of the nerve biology in leprosy. It is the only bacterium able to infiltrate the normal peripheral nerve. Although it does not have the virulent properties of *M. tuberculosis,* it may be regarded as a microorganism capable of interacting with the peripheral nerve components and of changing their metabolic pathways. The *M. leprae*-nerve relationship would be a fascinating subject for future research in leprous neuropathy.

In regard to neurotrophism, no specific leprosy alteration was detected. However, this does not rule out the possibility of neurotrophic disturbances in leprosy, since there are other types of neurotrophic factor receptors with their respective ligands

TABLE 4. *Equivalent NGFr-immunoreactivty of neural fibers in the cutaneous lesions of leprosy patients (L) and in the body region-, age- and sex-matched specimens of the control group (C); a semi-quantitative numerical estimate.*[a]

	epi		subepi		sg		dv		hf		apm	
	C	L	C	L	C	L	C	L	C	L	C	L
1	0	0	++	+	++	+	+	+	ND		++	+
2	0	0	++	+	++	++	+	0	+	++	+++	++
3	0	0	+	++	++	++	+	+	ND		0	++
4	0	0	++	+	ND		+++	+	+++	+	++	+
5	0	0	++	++	++	++	+	+	++	++	ND	
6	0	0	+	+	++	0	+	0	ND		ND	
7	0	0	+	+	++	++	+++	+++	ND		ND	
8	0	0	+	+	+	++	+	+	ND		ND	
9	0	0	+	+	+	++	+	+	ND		ND	
10	0	0	++	++	++	++	++	++	++	++	2	2
11	0	0	++	++	++	++	++	++	++	++	ND	
T	0	0	17	15	18	17	17	12	11	10	9	8

[a] Abbreviations of the histological cutaneous regions: epi = in the epidermis; subepi = in the subepidermal region; sg = adjacent to sweat glands; dv = adjacent to dermal vessels; hf = adjacent to hair follicle; apm = in the arrectores pili muscle. ND = Structures of this type were not found in any of the sections among either control or leprosy specimens, so that comparisons were Not Done; T = sum of the scores for each cutaneous region.

which were not investigated in this study ([13]). Anand, *et al.*'s report ([1]) indicates the need for more refined biochemical methods for the detection of nerve growth factors and their receptors in the tissues.

Our findings demonstrate the existence of a selective alteration of neuronal protein expression in the cutaneous innervation, which may or may not be directly related to the inflammatory infiltrate of early leprosy.

SUMMARY

We examined the immunohistochemical expression of the neuronal proteins NGFr, PGP 9.5, and NSE in cutaneous lesions of patients with early leprosy and in the skin of normal individuals. PGP 9.5- and NSE-immunoreactive nerve fibers were decreased in the skin of leprosy patients. This reduction was topographically unrelated to the early leprosy infiltrate. However, no difference in the expression of NGFr was found between the leprosy patient and normal groups. It was shown that there is a selective alteration in the expression of neuronal proteins in early leprosy lesions which seems to be unrelated to the inflammatory infiltrate in the initial stages of leprosy. Pathogenic mechanisms other than inflammation, which are intrinsic to the *Mycobacterium leprae*-nerve relationship, may thus contribute to the nerve damage in leprosy neuropathy.

RESUMEN

Examinamos la expresión inmunohistoquímica de las proteinas neuronales NGFr, PGP 9.5, y NSE, en las lesiones cutáneas de la lepra temprana y en la piel de

individuos sanos. En los pacientes con lepra encontramos una disminución de las fibras nerviosas que expresan las proteinas PGP 9.5 y NSE. Esta reducción no estuvo topográficamente relacionada con la localización de los infiltrados tempranos de la lepra. La expresión de la proteína NGFr fue similar entre los pacientes con lepra y los individuos sanos. Demostramos que hay una alteración selectiva en la expresión de las proteínas neuronales en las lesiones de la lepra temprana que no parece ester relacionada con el infiltrado inflamatorio en las etapas tempranas de la lepra. Probablemente otros mecanismos patogénicos, distintos a la inflamación misma, intrínsicos a la relación *Mycobacterium leprae*-nervio, puedan contribuir al daño en la neuropatía de la lepra.

RÉSUMÉ

Nous avons examiné l'expression immunohistochimique des protéines neuronales NGFr, PGP 9.5 et NSE dans les lésions cutanées de patients ayant une lèpre débutante et dans la peau d'individus en bonne santé. Les fibres nerveuses immunoréctives PGP 9.5 et NSE étaient en diminution ches les patients lépreux. Cette diminution n'était pas liée topographiquement à l'infriltrat de la lèpre débutante. Cependant, aucune différence dans l'expression de NGFr n'a été observée entre les groupes de patients lépreux et d'individus normaux. On a montré qu'il y a une modification sélective de l'expression des protéines neuronales dans les lésions de lèpre débutante, qui semble non liée à l'infiltrat inflammatoire des stades débutants de la lèpre. Les mécanismes pathogéniques autres que l'inflammation, qui sont intrinsèques à la relation nerf-*Mycobacterium leprae,* pourraient donc contribuer aux lésions nerveuses dans la neuropathie lépreuse.

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