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THE EXPRESSION OF NGFr AND PGP 9.5 IN LEPROSY REACTIONAL CUTANEOUS LESIONS

An assessment of the nerve fiber status using immunostaining

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ABSTRACT - The effects of reactional episodes on the cutaneous nerve fibers of leprosy patients was assessed in six patients (three with reversal reactions and three with erythema nodosum leprosum). Cryosections of cutaneous biopsy of reactional lesions taken during the episode and of another sample during the remission period were immunostained with anti-NGFr and anti-PGP 9.5 (indirect immunofluorescence). We found no significant statistical difference in the number of NGFr- and PGP 9.5-positive fibers between the reactional and post-reactional groups. A significant difference was detected between the number of NGFr and PGP 9.5-stained fibers inside of the reactional group of biopsy cryosections but this difference was ascribed to the distinct aspects of the nerve fibers displayed whether stained with anti-NGFr or with anti-PGP 9.5; NGFr-positive branches looked larger and so interpreted as containing more fibers. In addition, a substantial number NGFr-positive fibers were PGP 9.5-negative. No differences in the number of stained fibers among the distinct cutaneous regions examined (epidermis + upper dermis, mid and deep dermis) was detected. In conclusion, the number of PGP- and NGFr-positive fibers were not significantly different in the reactional and post-reactional biopsies in the present study. NGFr-staining of the nerve fibers is different from their PGP-imunoreactivity and the evaluation of the nerve fiber status on an innervated target organ should be carried out choosing markers for both components of nerve fibers (Schwann cells and axons).

KEY WORDS: nerve growth factor receptor, protein gene product 9.5, leprosy, neurotrophic factor.

Abbreviations: NGFr: nerve growth factor receptor; PGP 9.5: protein gene product; BB: borderline borderline patient; BL: borderline lepromatous patient; ENL: erythema nodosum leprosum; LL: lepromatous lepromatous patient; MDT: multidrug therapy; TIR: type I reactions; TIIR: type II reactions.

Expressão de NGFr e PGP 9.5 nas lesões cutâneas reacionais da hanseníase: uma avaliação do status das fibras nervosas utilizando imunomarcação

RESUMO - O efeito das reações hansenianas sobre a inervação cutânea de pacientes hansenianos foi avaliado em seis pacientes (três com reação reversa e três com eritema nodoso leprosum). Cortes congelados de biópsias de lesões cutâneas reacionais colhidas na ocasião da reação e de biópsias colhidas após a remissão do quadro reacional na mesma região ocupada previamente pela lesão foram marcados pela técnica de imunofluorescência indireta utilizando os anticorpos anti-NGFr e anti-PGP 9.5. Não foi encontrada diferença significativa na quantificação de fibras positivas para NGFr e para PGP 9.5 entre as biópsias colhidas durante a reação e as biópsias colhidas no período de remissão. Entretanto, no grupo de biópsias da reação houve uma significativa diferença entre a quantidade de fibras NGFr-positivas e as fibras imunomarcadas para PGP 9.5. Essa diferença contudo foi atribuída aos diferentes aspectos que a mesma fibra pode assumir quando marcadas com NGFr ou com PGP 9..5 separadamente. O presente estudo também mostrou que a avaliação das condições das fibras nervosas de um órgão deve ser realizada com marcadores para o axônio e para células de Schwann.

PALAVRAS-CHAVE: receptor do fator de crescimento neuronal, produto protéico do gen 9.5, lepra, fator neurotrópico.

Abbreviações: NGFr: nerve growth factor receptor; PGP 9.5: protein gene product; BB: borderline borderline patient; BL: borderline lepromatous patient; ENL: erythema nodosum leprosum; LL: lepromatous lepromatous patient; MDT: multidrug therapy; TIR: type I reactions; TIIR: type II reactions.

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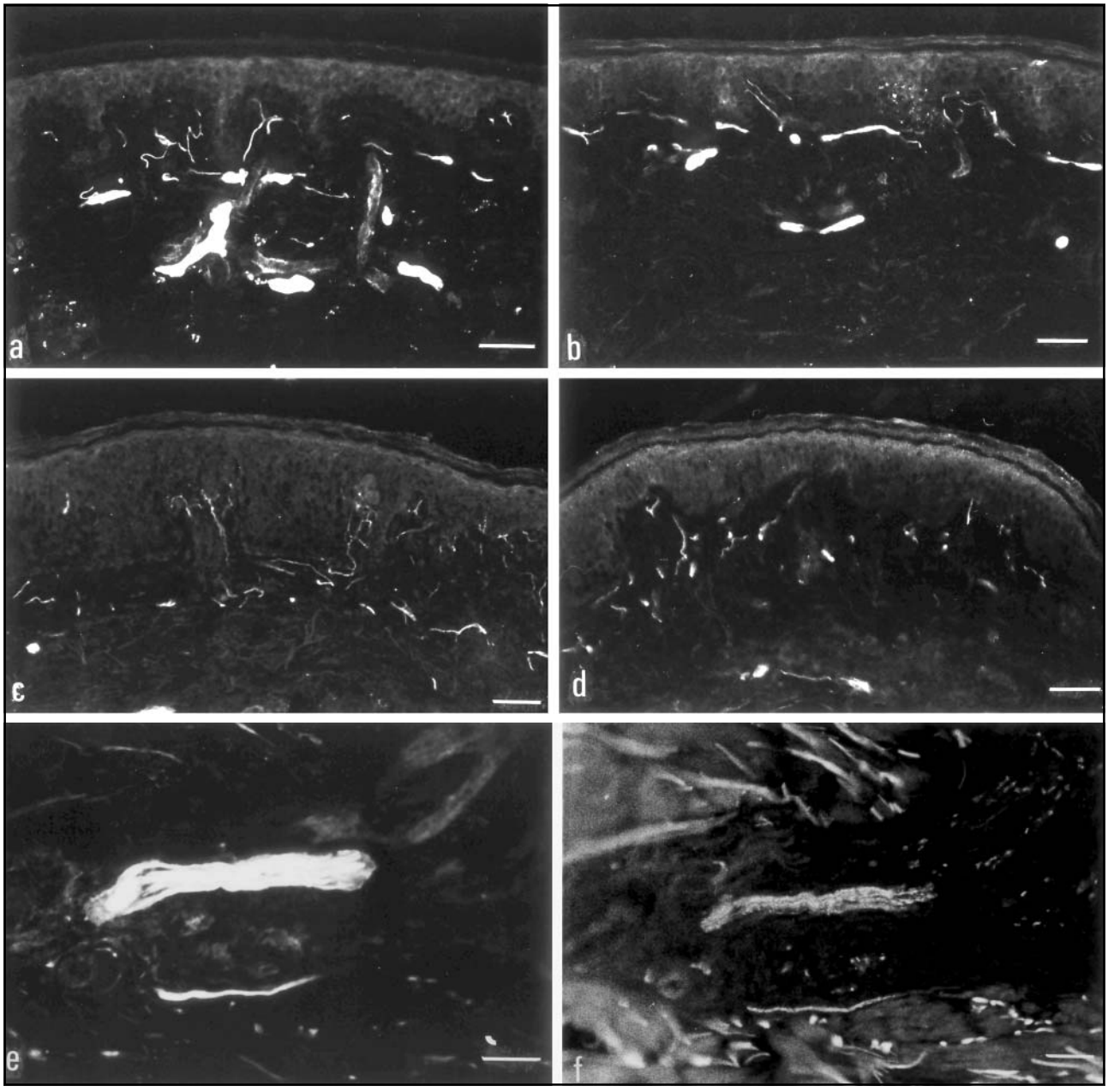
Leprosy reactions are acute recurrent clinical episodes which occur during the chronic course of the disease. There are two types of leprosy reaction: type I (TIR) and type II (TIIR; erythema nodosum leprosum = ENL, or multiform erythema) reactions[1]. TIR is characterized by the appearance of new lesions or reinfiltration of old ones, accompanied by neural symptoms and it is interpreted as a shift of the immunological status towards the tuberculoid pole (upgrading) or towards the lepromatous pole (downgrading)[1]. It occurs on the borderline patients of the immunopathologic spectrum of the disease[2]. The emergence of immature tuberculoid granulomas, increased lymphocytic infiltration, edema and vascular congestion with bacillary clearing on a borderline lepromatous infiltrate are the histopathological features of the TIR episode. TIIR (ENL) is clinically characterized by the appearance of subcutaneous nodules and general symptoms such as fever, malaise, ocular manifestations, edema and orchitis. On the histopathology, TIIR exhibits a characteristic neutrophilic infiltration upon a lepromatous cell infiltrate in the dermal-hypodermal boundary. Edema and nuclear fragmentation generally also occurs[1]. The triggering mechanism and the exact meaning of reactions have not been clarified yet, however exacerbation of the immune response has been detected during reactional episodes[3-7]. Leprosy reactions increase the morbidity of the disease due to nerve damage and impairment of nerve function[8]. These undesirable effects may even occur after the conclusion of multidrug therapy[5]. Paresthesia, hypoesthesia and paresia become progressively more intense if treatment is not promptly instituted[1]. Despite the decrease on the prevalence of leprosy in the world after the institution of the multidrug therapy, reactions continue to occur and may cause severe nerve damage to the patient under treatment or even after its conclusion.

The comprehension of leprosy pathogeny depends on an integrated knowledge of the pathobiology of the peripheral nervous system and the study on neuron survival, degeneration and regeneration which has remarkably progressed in the recent decade is within this large theme[9]. These pathobiological processes have been studied at molecular levels and so, the participation of neurotrophilic factors, cytoskeletal filaments, signaling molecules, membrane receptors, and degrading enzymes have been disclosed[10]. Neurotrophic factors or neurotrophins are represented by the NGF family and other family of molecules which bind to membrane receptors, mediating their effects. NGF receptors display two types of affinity: the high-affinity receptors or tropomyosin-related kinase receptors (trk A, trk B and trk C); each of them binding preferentially to a member of the nerve growth factor family) and the low-affinity receptor p75, which may enhance the affinity of tkr for NGF but does not mediate directly any neurotrophic effect[11]. Low-affinity NGFr (p75) may also directly mediate NGF-induced Schwann cell migration[12], the development of calcitonin-gene-related-peptide- and substance P-containing fibers[13] and it also plays a role in NGF-induced apoptosis[14]. P75 NGFr is present in non-neuronal cells but its biological activity on them is unknown[15]. The expression of nerve growth factor receptors by Schwann cells corresponds to its non-myelinating status which may be constitutive or induced in the regenerative process and the loss of NGFr, NCAM, GFAP and L1 expression corresponds to the achievement of a remyelinating activity on regenerating axons[16].

Neurotrophism in leprosy has been studied by Anand et al.[17], who detected depletion of NGF in the skin of leprosy patients using enzyme-linked immunoassay. Facer et al[18,19] related the lack of NGF neurotrophism to the decreased nerve function and decreased expression of an axonal sodium channel. Antunes et al,[20] have also compared between NGFr and PGP expression in the early macular cutaneous lesions of leprosy. Attempts to detect neurotrophic disturbances examining NGFr immunohistochemical expression were also carried out in peripheral neuropathies other than leprosy[19], and in amyotrophic lateral sclerosis[21]. On the other hand, protein gene product 9.5 (PGP 9.5) is widely utilized as a neuronal marker in studies of the peripheral nerve pathologies[22,23]. These studies have shown the extent of peripheral nerve damage in target organs, in diseases in which the peripheral nervous system was involved.

In the present study we evaluated the expression of NGFr (p75) and protein gene product 9.5 (PGP 9.5) in skin biopsies taken from patients under the reactional episodes and in the remission period. Regarding the two selected markers, NGFr is predominantly expressed by Schwann cells and on sympathetic and sensory neural crest-derived axons[9] in the normal skin and protein gene product, is a panaxonal marker utilized in the study of peripheral nerve fibers in target organs. NGFr is predominantly expressed by Schwann cells of the peripheral nerve, by the axons and PGP 9.5 immunoreactivity is displayed by axons so that both components of the fiber could be evaluated. We studied the effects of leprosy reactions on the cutaneous nerve fibers, analyzing some of their molecular components which

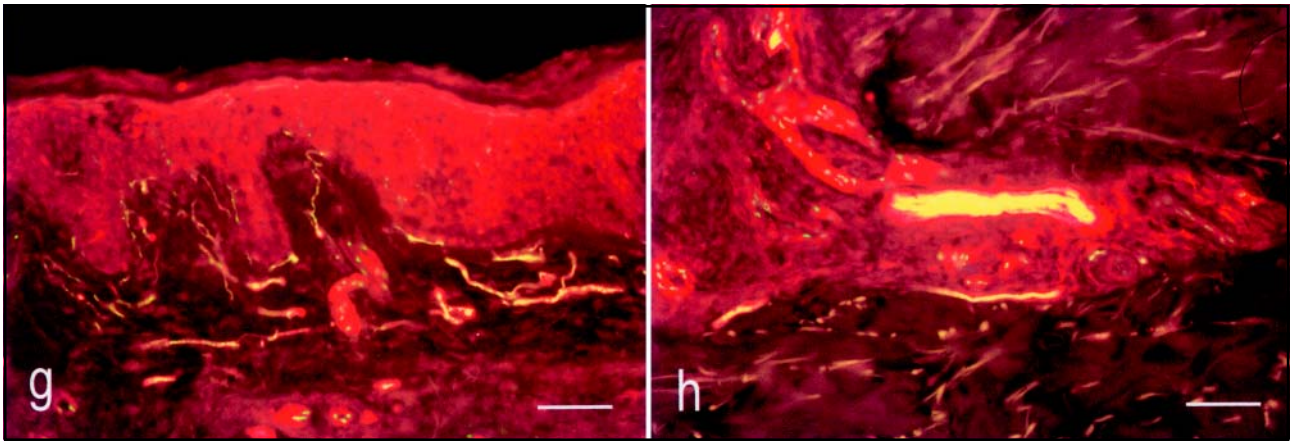


Figs a-f: NGFr immunoreactive fibers in a reactional (a) and in a post-reactional biopsy (b). The number and pattern of positive fibers were not significantly different in both groups. c-d: PGP 9.5 immunohistochemical expression in reactional (c) and post-reactional biopsies (d). The intensity and the pattern of immunoreactivity is not distinct between both sections. Note that the intensity of the immunoreactivity as well as the thickness of the positive fibers are lower than those shown in figures a and b, stained with anti-NGFr. This occurs due to the NGFr expression on perineurium and on Schwann cells together. e-f: NGFr (e) and PGP staining (f) of a nerve branch surrounded by inflammatory infiltrate (same field). Note that the NGFr-positive fibers are more intensely stained and also thicker than that shown with PGP staining.

are implicated in neural degeneration and regeneration considering that these two processes undoubtedly occur in leprosy[24].

METHOD

Six leprosy patients were selected for this study. Their clinical data are showed in Table 1. These patients had the diagnosis of leprosy confirmed in the leprosy outpatient service of the Oswaldo Cruz Institute. Leprosy patients are usually followed in this service during their treatment and along their post-discharge period. Three patients had TIR and the other three had TIIR. In this study, reactions were analysed as a single group because the number of each type of reaction was low. The procedures for selection of the patients were approved by the Ethics Committee for Research in Humans of Oswaldo Cruz Institute and the patients agreed to pariticipate in the study by signing an enlightened consent term.



Figs g-h: Double staining of NGFr and PGP showing the nerve fibers in the subepidermal region and a branch in the deep dermis. Green color corresponds to the PGP-positivity alone and reddish color to the NGFr-positivity. Yellowish color is the result of simultaneous NGFr- and PGP-positivity.

Table 1. Clinical data of the patients.

Patient	Age	Sex	Clinical form	MDT	Reaction	Skin lesion	Sensorial impairment	Paresthesia	Paresia	General symptoms	Time between reaction and remission
ERS	29	M	LL	MB	TIIR	Diffuse infiltration	Shins, knees, ankles	No	Intrinsic muscles of the hands	No	5 months
HJ	21	M	BL	MB	TIIR	Plaques/ nodules	No	No	No	No	1 month
JSS	16	M	BL	MB	TIIR	Plaque Infiltration of the ears	Hand, feet	Forearms, feet	Intrinsic muscles of the hands	Edema of the ankles	4 months
MPC	63	M	BL	MB	TIR	Plaques (diffuse)	Shin, right foot the hands	Feet	Intrinsic muscles of	Edema of ankles	1 month
MV	63	M	BB	MB	TIR	Foveolar plaques (diffuse)	Soles	Right elbow	No	No	1 month
ZAC	59	F	BL	MB	TIR	Plaques (diffuse)	Feet	No	No	No	5 months

BB, borderline borderline patient; BL, borderline lepromatous patient; LL, lepromatous lepromatous patient; MB, multibacillary patient; MDT, multidrug therapy for multibacillary patient; PB, paucibacillary patient; TIR, type I reaction; TIIR, type II reaction.

The selected patients were submitted to a first skin biopsy during the reactional episode and a second one during the remission period. The patients were considered to be in remission after the reactional lesions had decreased either in number or in degree of infiltration and all the general reactional symptoms had subsided. Both biopsies (reactional and post-reactional) were serially taken from close skin sites of the patient. The specific treatment for each kind of reaction (prednisone 1mg/kg of body weight/ day for TIR and thalidomide 200 mg/day for TIIR) was conveniently instituted for each patient.

The cutaneous biopsies were taken with a 6-mm punch and were fixed in a 4% paraformaldehyde solution containing 14% of saturated picric acid for two hours at 4°C, rinsed four times in 0.1 M phosphate buffer with 10% sucrose added, stored in liquid nitrogen, and sectioned on a Microm cryostat (Heidelberg, Germany). The sections were thawed onto pre-coated glass slides (Super

Frost®Plus, Menzel-Gläser, Braunschweig, Germany). The other half was processed for routine diagnostic procedures (hematoxylin-eosin and Wade staining).

Two non-adjacent sections for each biopsy were picked for the staining and counting of positive fibers and both biopsies (reactional and post-reactional) of each patient were processed in parallel for the immunostaining of NGFr and PGP. Double-staining with indirect immunofluorescence methods were utilized.

The primary antibodies selected for this study were rabbit anti-NGFr (Chemicon USA) and mouse anti-human PGP (UltraClone Cambridge) diluted in 0.01 M phosphate buffer containing 0.3% Triton X-100, in which the sections were incubated overnight at 4°C in a humid chamber followed by the incubation of fluorescein isothyocyanate (FITC)-conjugated donkey anti-mouse (1:160, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) together with LRSC-conjugated donkey anti-chicken (1:160, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Both were diluted in 0.01 M phosphate buffer containing 0.3% Triton X-100.

The sections were incubated with both primary antibodies overnight at 4°C in a humid atmosphere and control sections were incubated with corresponding normal sera instead, this was followed by the incubation with both second antibodies. The observation was performed using different excitation lights with a photomicroscope (Nikon, Tokyo, Japan). The immunohistochemical expression of NGFr and PGP markers were compared in both types of biopsies.

NGFr- and the PGP-stained nerve fibers were counted separately on the same field using the appropriate filter for the respective fluorochrome used. The frame utilized for taking microphotographs was employed as a standard field for counting nerve fibers. The immunoreactive nerve fibers inside six frames per section were counted. The frames were placed on the section according to distribution of two on the upper dermis, two on the middermis and two on the deep dermis. The objective lens utilized on the counting was the 20X objective matched with a 10X ocular. The nerve endings in the subepidermal region as well as the ones surrounding anexial structures, and dermal vessels were counted as individual fibers; average thick nerve branches on the mid dermis were estimated to have about forty-five fibers and the thicker branches in the deep dermis exhibiting the presence of perineurium were estimated to contain about seventy-five nerve fibers. This estimation was based on a previous ultrastructural visualization of cross sections of dermal nerve branches performed in other study[25].

The results of nerve fibers quantification were analyzed with the Statistica (Statsoft Inc. USA) software using the Wilcoxon's non-parametric paired analysis, Mann Whitney U and ANOVA tests.

Table 2. Number of NGFr- and PGP-positive nerve fibers in reactional biopsies (NGFrR and PGPR) and in post-reactional biopsies (NGFrPR and PGPPR) (6 frames/section, Magnif.: 200X).

Patient	Dermis	NGFrR	NGFrPR	PGPR	PGPPR
1	Upper	63,5	15,5	20	75,5
	Mid	133.5	52,5	46	0
	Deep	13,5	3,5	16	16
2	Upper	160	132	87,5	36
	mid	140,5	128	48,5	73
	Deep	140,5	94	40	20
3	Upper	160	174,5	96,5	82,5
	Mid	22	22	72,5	72,5
	Deep	94	94	102,5	102,5
4	Upper	1,5	94	0	116
	mid	121,5	135	12,5	135
	Deep	138	111	72	81
5	Upper	107,5	104,5	11,5	103,5
	mid	63,5	216	80	199
	Deep	354	120,5	377,5	56
6	Upper	34,5	41,5	26	26
	mid	50	79,5	31,5	31,5
	Deep	42	75	0	0

RESULTS

NGFr- and PGP- immunoreactive fibers were observed in all the reactional and post-reactional biopsies examined. The higher density of terminal fibers were exhibited on the subepidermal regions (Figs a, b, c, d) surrounding the anexial structures, microvessels, and among the smooth muscle cells of arrector-pillus muscles. Thick branches with NGFr-positive perineurial boundaries were seen in the mid and deep dermal regions (Fig a). No NGFr-immunoreactive fibers were seen in the epidermis but only PGP-positive fibers.

The number of NGFr- and of PGP- immunoreactive nerve fibers in the whole skin and per region of the skin sections, in the reactional biopsies were not significantly different from those in the post-reactional group. (Table 2) (Fig a-b, c-d).

The great majority of fibers were both NGFr- and PGP 9.5-positive, but they look more intensely stained and thicker when stained with anti-NGFr (Fig e-f). This more evident expression of NGFr was due to the immunoreactivity of perineurium, Schwann cells and axons together; the less intense PGP immunoreactivity instead, was ascribed to single axonal expression of this protein. In addition, few NGFr-stained fibers were PGP 9.5-negative in the same field.

As NGFr-positive branches look larger than the PGP-stained ones, we considered the former as containing more fibers than the latter, finding then, a higher number of NGFr- than of PGP-immunoreactive fibers inside of reactional but not in the post-reactional group (Table 2). This result however, will be commented and interpreted in the following section of this article.

No significant differences in the number of PGP 9.5- and of NGFr-immunoreactive fibers were found among the distinct cutaneous section regions counted inside each group (Table 2).

DISCUSSION

Basically we have found two results in this study: 1) the number of NGFr- and PGP-positive fibers were not significantly different in the reactional and in the post-reactional biopsies, 2) the total number of NGFr-positive fibers in the whole skin sections was significantly higher than that of PGP-immunoreactive ones in reactional but not in the post-reactional group of biopsies.

The first finding should be considered cautiously since comparison of samples with higher number of patients in each group could unveil a significant difference in the number of positive fibers. Nevertheless, we expected to find a higher number of NGFr- and PGP-positive fibers in the post-reactional group due to a supposed nerve regeneration which is reported to occur in leprosy patients' nerve trunks[26]. Miko however, this author remarks that the regeneration observed in the nerve trunks was not effective because the restoration of nerve function was not significantly achieved in the lapse of time (2 to 40 years) between the diagnosis of the patients and the beginning of his investigation. This implies that regenerating fibers, which are observed in the trunk may not reach the target organs, whick is the skin in the case of this study.

Expression of NGFr p75 on Schwann cells corresponds to a non-myelinating phenotype of this cell and also both neural crest-derived sensory and sympathetic neurons normally express this receptor[9]. Regeneration of nerve fibers induces increased NGFr expression on the axons and the reexpression of NGFr on denervated Schwann cells. The expected consequences of a leprosy reactional episode on the NGFr-immunoreactivity of peripheral nerve fibers therefore would be a decrease of this expression followed by an increased reexpression of this receptor by the Schwann cells and by the axons. In the present study we could not disclose any effect of the reaction on the number of NGFr and PGP 9.5-immunostained nerve fibers. This finding however, doesn't rule out other changes in molecular expression of nerve fibers.

Unfortunately it was not possible in this study to compare the number of the positive fibers in reactional biopsies with those of the pre-reactional state. This procedure could disclose a possible decrease of nerve fibers during reactional episode and a recovery of the their previous number in the remission stage.

The higher number of NGFr-positive fibers than that of PGP-positive ones in the same specimen of the reactional group of biopsies may be partially explained by the fact that distinct structures of the nerve fiber express the chosen markers. Therefore, NGFr is expressed on the Schwann cells, on perineurial cells and on the axons[27,28] while PGP 9.5 is only an axonal marker. This distinct pattern of expression confers to a bundle of fibers distinct appearances depending on wheter they were stained for NGFr or for PGP. NGFr-positive branches seem to be larger and more evident because of the axons, Schwann cells embracing them and the perineurial cells together express this receptor strengthening the intensity and thickening the area of the nerve branch immunoreactivity. PGP-positive fibers are thinner and comparatively less evident because only axons are stained. The estimation of the number of the nerve fibers based on the thickness of the positive nerve branches may mislead us to the conclusion in respect of the comparison of NGFr and PGP immunoreactivity in the same biopsy specimen. In addition, few of NGFr-positive fibers were PGP-negative, suggesting a selective molecular change of the fiber or axonal degeneration leaving surviving NGFr-positive Schwann cells. Using the methods of the present study, it was not possible to discern which of these two hypothesis correspond to reality. Immunoelectronmicroscopy with both anti-NGFr and PGP 9.5 would enlighten this point. Therefore, we could not state with the methods used in present study that there is really a higher number of NGFr- than of PGP-positive fibers in either group of biopsies.

The results of this investigation show that morphological and functional evaluation of nerve fibers should be carried out with markers for both Schwann cells and axons and also allow a reinterpretation of our previous findings[20] in which we reported a decreased expression of PGP 9.5 and NSE (neuron specific enolase) but not of NGFr on the nerve fibers in the biopsies of early macular lesions of leprosy patients. We thought that this occurred due to selective molecular change in the axons affected by leprosy,

not accounting the Schwann cell component of nerve fiber expressing NGFr. Under the light of Liang et al reports and also of the present study, we can state that PGP-negative fiber may retain its NGFr-positivity because of NGFr-expressing Schwann cells.

In conclusion, the number of PGP- and NGFr-positive fibers was not significantly different in the reactional and post-reactional biopsies in the present study. NGFr expression is different from PGP positivity on the nerve fibers and the evaluation of the nerve fiber status on an innervated target organ should be carried out choosing markers for both components of nerve fibers.

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