Pathology of Nerve Terminal Degeneration in the Skin

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Abstract. To characterize the pathology of epidermal nerve degeneration and regeneration, we investigated temporal and spatial changes in skin innervation of the mouse footpad. Within 24 hours after sciatic nerve axotomy, terminals of epidermal nerves appeared swollen and there was a mild reduction in epidermal nerve density (5.7 ± 2.8 vs 12.7 ± 2.2 fibers/mm, p < 0.04). Epidermal nerves completely disappeared by 48 hours (0.2 ± 0.2 vs 14.2 ± 0.9 fibers/mm, p < 0.001). Concomitant with the disappearance of epidermal nerves, the immunocytochemical pattern of the subepidermal nerve plexus became fragmented. At the electron microscopic level, the axoplasm of degenerating dermal nerves was distended with organelles and later became amorphous. Beginning from day 28 after axotomy, collateral sprouts from the adjacent saphenous nerve territory extended into the denervated area with a beaded appearance. They never penetrated the epidermal-dermal junction to innervate the epidermis. In contrast, 3 months after nerve crushing, the epidermis on the surgery side resumed a normal innervation pattern as the epidermis on the control side (10.3 ± 3.9 vs 10.6 ± 1.5 fibers/mm, p = 0.1). This study demonstrates the characteristics of degenerating and regenerating nerves, and suggests that successful reinnervation mainly originates from regenerating nerves of the original nerve trunks. All these findings provide qualitative and quantitative information for interpreting the pathology of cutaneous nerves.

Key Words: Axonal degeneration; Cutaneous nerves; Free nerve endings; Intraepidermal nerves; Nerve regeneration; Skin innervation; Unmyelinated axons.

INTRODUCTION

Evaluation of cutaneous nerve terminals in the epidermis of skin by immunocytochemistry provides a major advance in the study of peripheral sensory nerve disorders at the light microscopic level (1–4). Epidermal nerves are readily demonstrated with the neuronal marker protein gene product 9.5 (PGP 9.5), a ubiquitin carboxyl hydrolase. This approach offers an important measurement complementary to functional studies of cutaneous innervation in both humans and laboratory animals (2, 5–9). By taking advantage of skin biopsy, epidermal nerves can be quantified for diagnosis of sensory neuropathy (3, 10, 11). There is a significant reduction in the abundance of epidermal nerves in different types of sensory neuropathies (10, 12–14). Quantitation is mainly based on Wallerian degeneration, a final common pathway of nerve degeneration following mechanical, chemical, and toxic insults (15, 16). However, only limited studies have explored the issue of qualitative differences between normal degenerating and regenerating cutaneous nerves (3, 7, 13).

In rodents, degeneration of nerve terminals is usually completed within days (4, 8). The loss of cutaneous innervation following nerve injury has been demonstrated in a well-established experimental animal system (6, 17, 18). Only limited studies have examined ultrastructural features of individual nerves in the early phase of degeneration. Characterization of the pathology of degenerating and regenerating cutaneous nerves in the aforementioned system should provide important clues for clinical application of skin biopsy and new insights into potential mechanisms of nerve degeneration.

Fig. 1. Diagram of foot innervation in mice. This diagram shows the major innervation of the mouse hind foot from the sciatic and saphenous nerves. The sciatic nerve innervates the plantar side of the foot and the lateral aspect of the foot dorsum. The saphenous nerve innervates the medial aspect of the foot dorsum. The shaded area on the foot dorsum is innervated by both nerves. The circles depict footpads and the numbers indicate the relative positions of each pair of footpads. The first pair (1) is the most distal pair of footpads. The third pair (3) is the most proximal pair near the heel, with the second pair (2) localized between the first and third pairs.
Fig. 2. Temporal changes of epidermal nerve degeneration. Normal epidermis (A) and the epidermis denervated at various time points after sciatic nerve injury (B–F) were immunolabeled with protein gene product 9.5 (PGP 9.5). A: In control epidermis there are numerous PGP 9.5(+) nerves forming a dense subepidermal plexus (arrow). PGP 9.5(+) epidermal nerves have typical varicosities extending to the upper portion of granular layers. B: Within 1 day of sciatic nerve axotomy, epidermal nerves retract from the top of granular layers and terminal swelling becomes obvious (arrowheads). C: Epidermal nerves disappear by 2 days after axotomy of the sciatic nerve. PGP 9.5(+) immunoreactivity in the subepidermal plexus becomes fragmented (open arrowhead). D: Four days after denervation there are only degenerated axons in the subepidermal area. E: By day 7 of nerve injury there is no PGP 9.5(+) epidermal nerve nor PGP 9.5(+) dermal nerve. F: Twenty-eight days after nerve axotomy the epidermis remains denervated and the immunocytochemical pattern is similar to that in (E). Scale bar = 50 μm.

Restoration of neural functions after peripheral nerve injury depends on reconnection between nerves and their targets. Theoretically, reinnervation of targets can be achieved by regeneration from original nerve trunks, or by sprouting from neighboring nerves (19). Both mechanisms require different sets of specific molecules (20, 21). Variables influencing the capacity of regeneration include the types of nerve injury and the environment.
where nerve regeneration will take place (22–24). In mice, reinnervation of the cutaneous field occurs within weeks following nerve-crushing injuries (8, 18). The roles of regeneration and sprouting for proper reinnervation of the epidermis after denervation of long duration remain to be determined.

To address these issues, we investigated temporal changes of cutaneous nerve degeneration and regeneration, and characterized ultrastructural patterns of cutaneous nerves at the early stage of nerve degeneration.

**MATERIALS AND METHODS**

**Animal Surgery**

We used male 8-week-old ICR mice (35–40 g, National Taiwan University College of Medicine Animal Center, Taipei, Taiwan) for the following studies. Mice were anesthetized with chloral hydrate (40 mg/kg) for surgery. The sciatic nerve on the right side was transected at the thigh level (the sciatic nerve crush group). A segment (3–5 mm) of the sectioned sciatic nerve was removed, and the end of the proximal stump was sutured to the overlying muscle fascia and subcutaneous tissues to prevent the reconnection of the proximal and distal cut ends (4, 8). For each animal, the left sciatic nerve was sham-operated as the control. Three to 5 animals were sacrificed at different intervals after sciatic nerve axotomy: 1, 2, 3, 4, 5, 7, 10, 14, 28, and 90 days. To study reinnervation, additional groups of 5 mice were subject to crushing injury on the right sciatic nerve (the sciatic nerve crush group). These mice were sacrificed 90 days later for comparison with the axotomy group. Experiments adhered to the National Research Council Guide for the care and use of laboratory animals.

In mice, there were 3 pairs of footpads on the plantar side of the hindpaw (Fig. 1). The footpads were glabrous skin and were designated as the first, second, and third pairs. The areas immediately exterior to the footpads on the medial and lateral sides were pilary skin. The plantar side and the lateral aspect of the dorsal side (from the third to the fifth toes) were innervated by the sciatic nerve, and the medial aspect of the dorsal side was innervated by the saphenous nerve based on previous functional and morphological studies (Fig. 1) (8, 18, 25, 26). When we sampled tissues, each pair of pads with the interpad skin was processed together. In preliminary studies, we compared results of denervation among the 3 pairs of footpads, and the results were similar. Therefore, only findings of the first pair of footpads were reported.

**Immunocytochemistry**

For immunocytochemistry on freezing microtome sections (3), animals were fixed by intra-cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The skin areas innervated by the sciatic nerve, including the glabrous skin (footpads) and the pilary skin (area lateral to the pads), were fixed for another 6 hours (h), and then changed to PB for storage. After thorough rinsing in PB, samples were cryoprotected with 30% sucrose in PB overnight. Sections perpendicular to the dermis of 30 μm were cut on a sliding microtome. Sections from each tissue were labeled sequentially and stored with anti-freeze at −20°C. To ensure adequate sampling, every fourth section for each tissue was chosen for PGP 9.5-immunostaining. The sections were treated with 0.5% Triton-X 100 in 0.5 M Tris buffer (pH 7.6) (Tris) for 30 min and processed for immunostaining. Briefly, the sections were quenched with 1% H₂O₂ in methanol, and blocked with 5% normal serum of appropriate species in 0.5% nonfat dry milk in Tris. The sections were incubated with rabbit antiserum to PGP 9.5 (UltraClone, UK, 1:1,000 diluted in 1% normal serum in Tris) for 16–24 h. After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit IgG for 1 h and the avidin-biotin complex (Vector, Burlingame, CA) for another hour. The reaction product was demonstrated by 3,3’-diaminobenzidine (DAB, Sigma, St Louis, MO).

**Quantitation of Epidermal Innervation**

Epidermal innervation was quantified according to modified protocols in a coded fashion (3, 8, 27). PGP 9.5-immunoreactive nerves in the epidermis of each footpad were counted at a magnification of 40× with an Olympus BX40 microscope (Shibuya-ku, Japan). Each individual nerve with branchings inside the epidermis was counted as one. For epidermal nerves with branchings in the dermis, each individual nerve was counted separately. The total length of epidermis along the upper margin of stratum corneum in each footpad was measured with the Image-Pro PLUS system (Media Cybernetics, Silver Spring, MD). Epidermal nerve density was therefore derived and expressed as the number of fibers per mm of epidermal length. Every fourth section for each tissue was quantified, and there were 3 sections for each footpad.

![Graph showing quantitation of epidermal nerve degeneration.](image-url)
Fig. 4. Normal and degenerating dermal nerves. Normal (A) and denervated (B–D) footpads were immunocytochemically labeled with protein gene product 9.5 (PGP 9.5) to illustrate dermal nerves. A: In the normal dermis, PGP 9.5-immunoreactive dermal nerve trunks appear with a linear pattern and dense staining intensity. B: Within 24 h of axotomy, PGP 9.5(+) dermal nerves have the same appearance as normal dermal nerves in (A). C: Seventy-two hours after nerve injury, dermal nerves undergo degeneration and have a globular appearance, reflecting degenerated axons and myelin ovoid formation. (D) Seven days post-surgery, all axonal staining disappears. In the dermis there is only faint staining on the membrane surface of denervated Schwann cells along the border of dermal nerves. Scale bar = 20 μm.

Ultrastructural Study

Mice were perfused with 5% glutaraldehyde in PB. The hind paws of both control and denervated sides were dissected. Tissues stayed in the same fixative overnight. After rising in PB, tissues were postfixed in 2% osmium tetraoxide for 2 h, dehydrated through graded ethanol, and embedded in Epon (28). Semithin sections were stained with toluidine blue. Selected areas were thin-sectioned and observed under a Hitachi electron microscope.

Statistical Analysis

For quantitation of epidermal innervation, groups of 5 animals were used at 1, 2, 7 days and 3 months after sciatic nerve axotomy and 3 months after sciatic nerve crush. The data were presented as mean ± SD. Analyses included t-test and Wilcoxon rank sum test between the control side and the experimental side at each time point with SPSS for Windows (version 6.1, SPSS Inc., Chicago, IL) and GraphPad Prism (version 2.01, GraphPad Software Inc., San Diego, CA). Any difference with p < 0.05 was considered statistically significant.

RESULTS

Temporal Changes of Cutaneous Nerve Degeneration

In a normal footpad, typical intraepidermal nerves had a varicose appearance extending to granular layers of the epidermis (Fig. 2A). Terminals of epidermal nerves became swollen within 24 h after sciatic nerve axotomy, and many of the epidermal nerves retracted from the granular layers (Fig. 2B). Quantitatively, there was a significant reduction in epidermal nerve density (5.7 ± 2.8 fibers/mm on the denervated side vs 12.7 ± 2.2 fibers/mm on the control side, p < 0.04, Fig. 3). Epidermal nerves no longer existed by 48 h (Fig. 2C, 0.2 ± 0.2 fibers/mm vs 14.2 ± 0.9 fibers/mm on the control side, p < 0.001, Fig. 3). Only PGP 9.5(+) spots were seen in the subepidermal plexus. Beginning at postaxotomy day 4, immunoreactivity for subepidermal plexuses disappeared altogether (Fig. 2D–F).

In the dermis, PGP 9.5(+) dermal nerves looked the same as normal dermal nerves within 24 h of sciatic
Fig. 5. Temporal changes of sweat gland innervation after sciatic nerve axotomy. To investigate sweat gland innervation, we immunostained normal skin (A) and denervated skin (B–F) with protein gene product 9.5 (PGP 9.5). A: In normal sweat glands, the innervation appears interlaced around the ductal and secretory portions of sweat glands. B: Within 24 h of nerve injury, the pattern of sweat gland innervation is similar to that in (A). C: By 48 h, the innervation of sweat glands disappears and the staining for remaining nerve trunks becomes discontinuous. D: The PGP 9.5-immunoreactivity in the sweat gland area is not recognizable 4 days after denervation. E: Seven days after nerve injury, the immunocytochemical pattern of sweat gland innervation is similar to that of day 4 denervation. F: Twenty-eight days post-surgery sweat glands remain denervated. There are only some discontinuous PGP 9.5-immunoreactive dots between sweat glands, which may reflect sprouting from neighboring nerve territories. Scale bar = 20 μm.
Fig. 6. Ultrastructural characterization of degenerating cutaneous nerves. A: By 24 h after sciatic nerve axotomy, there were occasional degenerating epidermal nerves between adjacent keratinocytes containing electron-dense keratohyaline granules (k). These axons are swollen with disrupted axonal membrane and the axoplasm becomes amorphous (arrow), representing disintegration of the axonal cytoskeleton. B: Within 24 h of sciatic nerve axotomy, unmyelinated axons in the subepidermal areas undergo degeneration (arrow) and some have intact axonal membrane (arrowhead). C: By 48 h of axotomy, most of unmyelinated nerves in subepidermal areas are degenerated (arrows). Only scanty of unmyelinated nerves have recognizable axonal membrane (white arrowhead). However, the axoplasm in these axons is amorphous and organelles are swollen. Scale bars: A = 0.67 μm; B, C = 1 μm.

Fig. 7. Reinnervation of skin 3 months after sciatic nerve crushing compared with the denervated skin 3 months after sciatic nerve axotomy. To compare patterns of innervation after different surgical procedures, we immunostained the skin with protein gene product 9.5 (PGP 9.5) from animals in the crushed-nerve group (left panel: A, C, E, and G) and the axotomy group (right panel: B, D, F and H). A: PGP 9.5(+) nerves appear in the epidermis and in the dermis, similar to those in the control footpads. B: There is no sign of reinnervation in the skin 3 months after axotomy. C: In the crushed-nerve group there are epidermal nerves and some of them reach the top of the granular layers. D: In the axotomy group, only discontinuous PGP 9.5-immunoreactivity parallels the subepidermal area. No epidermal nerve is discernible. E: Sweat gland innervation resumes the interlaced appearance after nerve regeneration. F: After permanent axotomy, only discrete dots of PGP 9.5-immunoreactivity appear near sweat glands. G: In the nerve-crushed group, PGP 9.5(+) dermal nerves appear normal like the control nerves in Figure 4A. H: In the axotomy group, only discontinuous PGP 9.5(+) dots are in the dermis. Scale bars: A, B = 200 μm; C, D = 50 μm; E–H = 23 μm.
Fig. 8. Quantitation of epidermal reinnervation. Epidermal reinnervation 3 months after sciatic nerve injury was quantified with 5 animals for each group. Epidermal nerve density is expressed as fibers/mm of linear epidermis. For the sciatic nerve axotomy group (Axotomy), the epidermis remains denervated (0.2 ± 0.2 fibers/mm, open bar) compared with the control (11.0 ± 3.5 fibers/mm, filled bar). In the sciatic nerve crush group (Crush), the epidermal nerve density of the previously denervated skin (10.3 ± 3.9 fibers/mm) is similar to that of the control skin (10.6 ± 1.5 fibers/mm, p = 0.1). Statistical analysis is based on t-test between the denervated epidermis and the control epidermis for each time point. ** < 0.001.

The time course and pattern of degeneration of nerves innervating sweat glands were similar to those of epidermal nerves. There was no distinguishing change within 24 h (Fig. 5A, B). By 48 h, the innervation of sweat glands disappeared and the staining for remaining nerve trunks became discontinuous (Fig. 5C). The PGP 9.5-immunoreactivity in the area of sweat glands was no longer recognizable during the period of denervation (Fig. 5D–F).

**Ultrastructural Appearance of Cutaneous Nerve Degeneration**

To study ultrastructural characteristics of degeneration in the terminal region of cutaneous nerves, we examined epidermal nerves and subepidermal plexuses in the early phase of degeneration. By 24 h of axotomy, the axoplasm of epidermal nerves became amorphous with disrupted axonal membrane, representing disintegration of the axonal cytoskeleton (Fig. 6A). In subepidermal area, some of unmyelinated axons had intact axonal membrane and others were undergoing degeneration (Fig. 6B). Within 48 h, disintegration of axonal cytoskeleton and swelling of organelles became prominent features. Only scanty unmyelinated axons continuing degeneration were recognizable (Fig. 6C).

**Reinnervation of the Skin after Sciatic Nerve Crush**

To investigate pathological characteristics of regenerating nerves, we compared the results of sciatic nerve crush with those of permanent sciatic nerve axotomy. Three months after nerve injury, the immunocytochemical pattern of the crush group was substantially different from that of the axotomy group (Fig. 7A, B). In the crush group, PGP 9.5(+) nerves appeared in the epidermis and dermis, similar to those in the control footpads (Fig. 7A, C, E, G). There was, however, no sign of reinnervation in the epidermis and dermis 3 months after sciatic nerve axotomy (Fig. 7B, D, F, H).

In the crush group, some of the epidermal nerves reached the granular layers (Fig. 7C). In the axotomy group, discontinuous PGP 9.5-immunoreactivity appeared in the subepidermal area, but these nerves never penetrated the epidermal-dermal junctions (Fig. 7D). Sweat gland innervation resumed and dermal nerves reappeared in the crushed-nerve group, with the same appearance as those in the normal skin (Fig. 7E–G). When nerve regeneration was retarded by permanent nerve axotomy, there were much fewer regenerating nerves in the dermis. The reinnervating nerves had a beaded appearance with axonal swellings. These included nerve terminals innervating the sweat glands, dermal nerve trunks, and subepidermal plexuses (Fig. 7D, F, H).

The influence of sciatic nerve axotomy and sciatic nerve crush on epidermal reinnervation was demonstrated by quantitation of epidermal nerves (Fig. 8). The number of epidermal nerves was negligible on the axotomized side 3 months after sciatic nerve axotomy (0.20 ± 0.20 vs 11.0 ± 3.5 fibers/mm, p < 0.001). In contrast, 3 months after sciatic nerve crush, epidermal nerve densities were similar between the control side and the experimental side (10.6 ± 1.5 vs 10.3 ± 3.9 fibers/mm, p = 0.1).

We further investigated clues of unsuccessful reinnervation of the epidermis after permanent sciatic nerve axotomy. This was based on the results from 28 days and 3 months after permanent nerve axotomy (Fig. 9). Growing nerves originated from the neighboring saphenous nerve and extended into the adjacent footpad in the sciatic nerve territory (Fig. 9A). At higher magnification, these nerves approached the epidermis of the footpad (Fig. 9B, C). None of the collateral sprouts extended beyond the epidermal-dermal junction after permanent nerve injury (Fig. 9C, c.f. Fig. 7D).

**DISCUSSION**

The present study demonstrates the early pathology of cutaneous nerve terminal degeneration, as well as the ultrastructural characteristics of degenerating nerves. Temporal and spatial patterns of nerve degeneration provide important information not only for understanding the mechanisms of Wallerian degeneration, but also for interpreting the findings of skin biopsies.

**Degeneration of Nerve Terminals in the Epidermis**

The depletion of intraepidermal nerves within 24–48 h suggests that nerve terminal degeneration is an early event, consistent with the finding of motor nerve terminal
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from the neighboring saphenous nerve territory was immuno-

stained with protein gene product 9.5 (PGP 9.5). The tissue was

sampled 28 days after nerve axotomy. A: PGP 9.5(/H11001

epidermal and dermal nerves are in the epidermis of the saphenous nerve

territory. Dermal nerves extend into the neighboring dermis. B:

At higher magnification, dermal nerves approach the epidermis

of the footpad. C: Collateral sprouts parallel the epidermal-der-

mal junction but never ascend in the epidermis. Scale bars: A

= 200 μm; B, C = 20 μm.

Cutaneous Reinnervation by Regenerating Axons

Both axonal regeneration and collateral sprouting contribute to reinnervation of the denervated skin. The overall outcome depends on types of nerves and lesions (23, 24, 39) and guidance molecules (19, 40–43). Collateral sprouts from neighboring saphenous nerves appear as early as 2 weeks after nerve injury. However, growth cones of these sprouts only extend laterally into their neighboring area for a limited distance. Despite approaching the epidermal-dermal junction, collateral sprouts fail to cross the basement membrane of the epidermis and ascend in the epidermis as normal intraepidermal nerves do. These findings extend previous observations (22) and provide a morphological basis for investigating the territory of nerves complementary to functional dermatome mapping (44). After the nerve-crushing injury, regenerating nerves from the sciatic
degeneration (24, 29). Epidermal nerve terminals become

swollen within 24 h of axotomy at the light microscopic level. This early sign of degeneration may reflect a blocking of axonal transport, and offers an ultrastructural illustration of the degenerating nerves in neuropathy (3, 13). Within 48 h of sciatic nerve axotomy, no more epidermal fibers exist. Some of the dermal nerves remain visible immunocytochemically with intact organization at the ultrastructural level. These findings indicate that the disappearance of PGP 9.5(+) epidermal nerves is not simply due to a difference in the clearance of PGP 9.5 between epidermal nerves and dermal nerves. Several mechanisms account for the faster degeneration of nerve terminals. For example, the temperature in the nerve terminal regions is lower than that of the proximal part of damaged nerves (16, 30, 31). Alternatively, calcium influx may mediate earlier nerve degeneration in nerve terminals (32). The presence of calcium channels in motor nerve terminals and calcium-dependent proteolysis may facilitate nerve terminal degeneration (16, 33). Axonal degeneration after rhizotomy illustrates different speeds of degeneration in different portions of the same nerve. Degeneration of nerve terminals in the region of the nucleus gracile is faster than that in the region of the dorsal column; both are the same central processes of dorsal root ganglion neurons (34, 35). Whether degeneration of sensory terminals shares the same mechanism remains an open issue. The presence of calcium-binding proteins in the dorsal root ganglion, whose peripheral processes constitute the cutaneous nerves, suggests that calcium may play a role in nerve degeneration and regeneration (36–38).

Fig. 9. Collateral sprouting after sciatic nerve axotomy. The footpad in the sciatic nerve territory with adjacent skin

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nerve trunks easily penetrate the basement membrane and terminate in the epidermis.

Regenerating nerves arising from the original nerve trunks appear to be the major source of successful reinnervation of previous denervated epidermis. Why does the sciatic nerve reinnervate the previously sciatic territory better than the saphenous nerves? Several lines of evidence suggest that nerve-target connections and territory integrity could exert complicated interactions. Neurotrophin requirement is different for collateral sprouting and regenerating growth. Collateral sprouting is dependent on endogenous nerve growth factor and regeneration from original nerve trunks is independent of nerve growth factor (20, 21). In acute stage of denervation, the content of nerve growth factor in the skin is up-regulated (45). However, the content of nerve growth factor in the long-term denervated skin is down-regulated (46). It remains to be determined whether there are factors inhibiting the extension of neurites into the aberrant territory as documented in the central nervous system, for example, the myelin-associated glycoprotein (42, 47–49). The establishment of nerve-target connection requires correct guidance cues, for example, attractive and repulsive molecules (41, 50, 51). These signals are mediated through cyclic nucleotides (52). Neurotrophins modulate the response of growth cones to guidance molecules (53). Further studies will be necessary to determine the contribution of individual factors and their interactions to successful reinnervation of the skin after nerve injury.

Characteristics of Degenerating and Regenerating Nerves

Recent applications of immunocytochemical staining of skin biopsies open a new field for evaluating sensory neuropathies. Much of the work has been focused on the quantitative features of intraepidermal nerve fibers (10, 11). A further and critical issue in applying such a technique is the morphological recognition of degenerating and regenerating nerves. Normal epidermal nerves have typical varicosities. Epidermal nerve terminals become swollen in the early degeneration phase. In contrast, growing axons from collateral sprouts have a beaded appearance. These characteristics provide the morphological basis for identifying sensory nerve pathology in skin biopsies (13, 54–57).

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