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Pathology and Quantitation of Cutaneous Innervation

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OVERVIEW

The validity of skin biopsy/blister for assessing the extent of cutaneous innervation, and especially epidermal nociceptors, has now been established. These techniques provide a reliable and reproducible means of assessing the numbers of C-fiber nociceptors. Easily applicable and robust quantitation of the dermal innervation, sweat glands, hair shafts, and other potentially instructive nerve fiber populations remains to be developed.

Skin biopsy/blister methods have opened new opportunities to learn about the response of unmyelinated nerve fibers to a number of disease entities and experimental conditions. The minimally invasive nature of the biopsy, blister, and epidermal sheet techniques makes it possible to study reactions of unmyelinated nerves to experimental conditions directly in human subjects. The methods are useful for diagnosis and may find a place in the longitudinal evaluation of disease progression. An area of particular interest is the potential value of serial skin biopsies in clinical trials of agents that are intended to promote regeneration of nerve fibers.

Evaluation of unmyelinated cutaneous nerves obtained by skin biopsy has emerged as a useful method to diagnose and study peripheral nerve disorders.[27,46,48,52,63] Until recently, work with peripheral nerves was restricted to studies of myelinated nerve fibers because methods available for assessment of small, unmyelinated fibers were limited. With standard electrodiagnostic studies, unmyelinated fibers remain "invisible" because nerve conduction studies assess only large sensory and motor fibers. Even assessment by sural nerve biopsy is problematic; electron microscopy is required to visualize unmyelinated fibers and nerve biopsy only gives a "window" into one location along the nerve at a single time point. Finally, from a functional standpoint,

cutaneous sensation is transduced by identifiable nerve fibers that enter their "targets" in the skin; these cannot be identified by nerve biopsies. These limitations and the observations that individuals with sensory neuropathies have spontaneous acral neuropathic pain with marked allodynia stimulated development of methods to identify and quantify unmyelinated nerves in the skin.

Early studies of cutaneous nerves obtained by punch skin biopsy defined the density and distribution of Meissner's corpuscles and their myelinated nerves in different age groups and in several hereditary disorders. Unmyelinated nerves were not visualized.[7,20] The development of sensitive immunohistochemical techniques offered a new approach to identify and quantify small sensory nerves. In particular, immunostaining of skin biopsies for protein gene product 9.5 (PGP 9.5), a neuronal ubiquitin carboxy-terminal hydrolase,[106] has been used by a number of groups to visualize the subpapillary plexus of small myelinated and unmyelinated nerve fibers.

Epidermal nerve fibers have received the greatest scrutiny, mainly because they appear to be early indicators of neuropathy and adequate samples can easily be obtained for quantitation.[18,45,63,110] These fibers include both Aδ and C fibers that convey "slow" nociception. We developed robust normative data,[61] and have demonstrated a usually distally dominant pattern of epidermal nerve fiber loss in diabetic neuropathy,[47] human immunodeficiency virus (HIV)–associated sensory neuropathies,[82] and idiopathic small fiber sensory neuropathies.[35] In patients with severe neuropathy, decreased epidermal densities are most severe distally, with less marked reductions found at progressively more rostral levels and prominent predegenerative axonal swellings identified even at asymptomatic sites. In fact, abnormalities of cutaneous innervation are found in some individuals with normal tendon reflexes at the ankles, normal sural nerve action potential amplitudes, and normal quantitative sensation tests (QSTs). Epidermal denervation also occurs in individuals with spontaneous allodynic pain.[34,78] While the precise structural correlates of allodynia remain uncertain, it can clearly occur even with marked depletion of Aδ and C fibers in the skin.

The association of neuropathic pain with epidermal nerve loss is, *en face*, paradoxical because nerve fiber loss is traditionally related to loss of sensation without pain. Neuropathic pain has been correlated with epidermal nerve loss in small fiber sensory neuropathy, postherpetic neuralgia, HIV-associated sensory neuropathies, and diabetic neuropathy. Explanations for this apparent paradox include possible changes in both peripheral and central nervous systems. In peripheral neuropathy caused by persistent action of an etiologic agent, continuous spontaneous pain can result from ectopic discharges in peripheral nociceptive fibers.[77] Alternatively, sensitized nociceptors responding with reduced threshold to weak stimuli that normally evoke touch, warm, or cool sensations also evoke pain (hyperalgesia). Capsaicin-induced mechanical and heat hyperalgesia is one example. Another is the neuronal excitability that results from increased voltage-gated Na^+ currents after exposure to serotonin, prostaglandin E_2, or adenosine, agents that produce tenderness or hyperalgesia.[26] Likewise, products of nerve degeneration might increase nerve excitation by unmasking proteins normally hidden from immune surveillance (e.g., P_0, P_2). Resultant immune cell activation exposes surviving nerves to inflammatory cytokines and other substances that alter threshold to stimuli.[111] There is growing evidence that injury to nociceptors leads to increased firing not only in injured axons but also in uninjured neighboring axons[115] and perhaps in dorsal root ganglia neurons at an adjacent level.[4] In fact, selective injury of a ventral root causes hypersensitivity of C fiber afferents in an adjacent root.[116] Subsequent activation of nociceptors is believed to produce central sensitization and secondary hyperalgesia. One mechanism is by central terminals of injured Aδ fibers that develop nerve sprouts from deep dorsal horn lamina into superficial laminae. If these synapse with nociceptive neurons, the neurons could become susceptible to activation by otherwise innocuous peripheral stimuli.[114] In addition, a shift of descending modulation from inhibition to facilitation can contribute to hypersensitivity.[84]

ANATOMIC FEATURES OF THE SKIN

Skin separates the external environment from internal tissue while providing sensory input, protection from pathogens and allergens, and a water barrier. The layered structure of skin imparts characteristics essential to these functions (Fig. 34–1). The distinct dermal and epidermal layers of skin comprise the major compartments of cutaneous structure. The epidermis is the topmost living layer of skin and consists of layers of keratinocytes that are replaced in approximately a 28-day cycle in humans as they are desquamated to form the cornified surface layer of dead cells that provides the water barrier. Intermixed among the vital keratinocytes are basal melanocytes, dendritic Langerhans cells, and sensory nerve terminals. The epidermis is separated from the dermis by the dermal-epidermal basement membrane. The area of dermis immediately internal to the epidermis, the papillary dermis, contains a vascular plexus with periodic capillary loops that reside within dermal protrusions into the epidermis called "dermal papillae." Within the matrix of the dermis, cutaneous structures include hair follicles with associated arrector pilorum muscles and sebaceous glands, blood vessels, nerve bundles, and sweat glands (Fig. 34–1).

The nerves to skin arise from sensory and motor neurons residing in dorsal root ganglia and sympathetic ganglia. Bundles of nerves enter the skin deep in the dermis and course toward the skin surface, giving off axons to

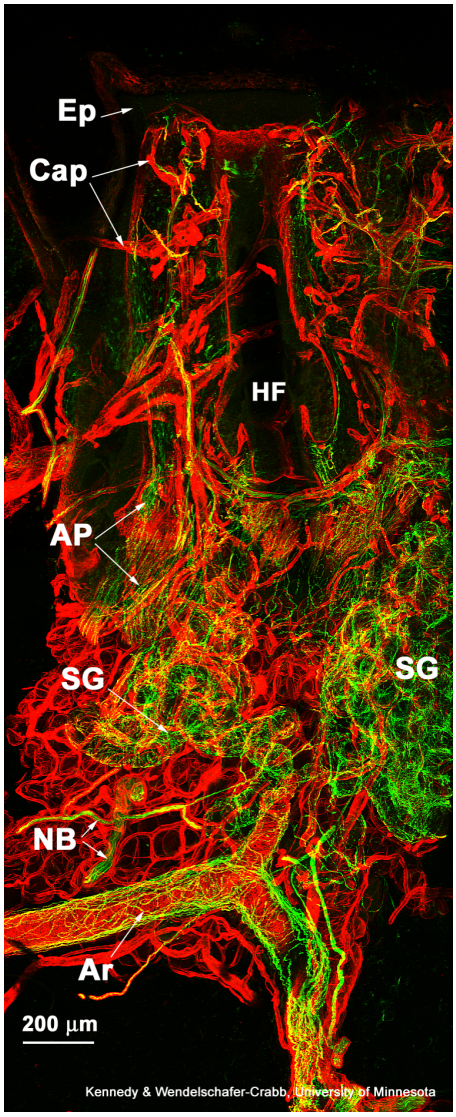

FIGURE 34–1
Human skin innervation and vasculature. Confocal image of 150-µm-thick section of human skin immunostained to demonstrate nerves (PGP 9.5—green and yellow) and basement membrane (type IV collagen—red). Image is a projection of 31 5-µm optical sections acquired with a 10 × objective lens. Basement membrane outlines cutaneous structure, including the basement membrane separating the dermis from the epidermis (Ep), capillary (Cap) loops of the papillary dermis and vasculature throughout the dermis, the central hair follicle (HF) and associated sebaceous gland, arrector pili muscles (AP), and sweat glands (SG). Nerve bundles (NB) arise from the deep dermis to innervate cutaneous structures. Characteristic nerve networks associated with arteries (Ar), sweat glands, arrector pili muscles, the hair follicle, and the subepidermal neural plexus provide sufficient morphologic criteria for identification of these structures based on their innervation patterns. *See Color Plate*

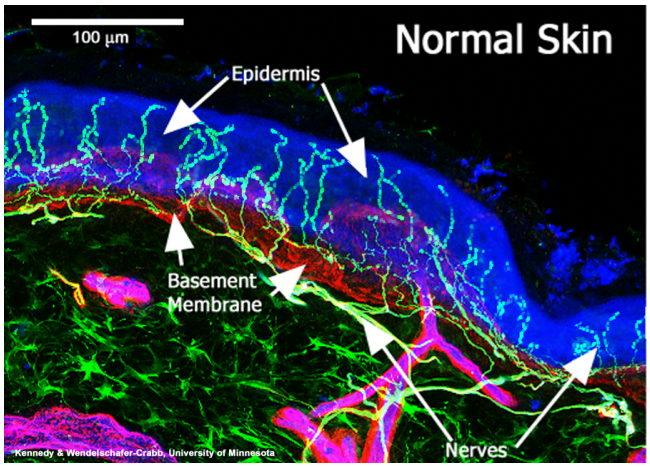

FIGURE 34–2
Normal human epidermal and papillary dermis innervation. Nerves are localized with antibody to PGP 9.5, and basement membrane is demarcated with antibody to type IV collagen. Vasculature is labeled with *Ulex europaeus* agglutinin type I. Epidermal nerve fibers appear aqua and lie within the blue epidermis (E). The subepidermal neural plexus (SNP) appears green or yellow. The dermal-epidermal junction appears red. Capillaries (C) appear magenta. Nerve fibers (green and aqua) course in bundles through the dermis and branch in the papillary dermis to form the subepidermal neural plexus. Fibers arise from this plexus and penetrate the epidermal-dermal basement membrane to enter the epidermis. Note that some non-neuronal fibroblasts appear green. Epidermal nerve fibers are abundant and uniformly distributed in normal human skin. *See Color Plate*

innervate the associated end organs. Unmyelinated nerve fibers comprise the vast majority of cutaneous innervation to the above dermal structures. The few myelinated nerve fibers terminate at hair follicles, Meissner's corpuscles, and Merkel complexes. When the vertically oriented nerve bundles enter the superficial papillary dermis, they form a horizontal subepidermal neural plexus (Fig. 34–2). Epidermal nerve fibers branch from this plexus and, while penetrating the dermal-epidermal basement membrane to enter the epidermis, they lose their Schwann cell ensheathment and collagen collar.[12] Within the epidermis they extend between keratinocytes to the surface of the vital epidermis. Autonomic nerves to sweat glands enwrap the coiled sweat tubules in such a dense pattern that they virtually cover much of the surface (see Fig. 34–1, SG). The autonomic innervation of the arrector pilorum muscles is easily distinguished within the muscle by a characteristic wavy pattern. The complex innervation pattern of hair follicles is composed of both myelinated and unmyelinated fibers, with specialized nerve endings at the base and along the shaft of the hair.[22,28,85] Fine unmyelinated nerve endings form a meshlike network covering larger arteries in the deep dermis, but only one or two nerves are on the more superficial smaller arterioles (see Fig. 34–1, Ar).

Epidermal nerve fibers are constantly remodeling in concert with the migration and shedding of keratinocytes during the turnover of epidermal layers. In addition,

epidermal nerve fibers respond to injury by regenerative regrowth and collateral sprouting. The neurotrophic requirements of epidermal nerve fibers have been studied, and at least three trophic factors have been found to affect C-fiber nociceptors: nerve growth factor (NGF), glial cell line–derived neurotrophic factor (GDNF), and insulin-like growth factor type 1. The neurons that depend on NGF, approximately half the small sensory neurons in rat dorsal root ganglia, express the high-affinity NGF receptor TrkA as well as the low-affinity receptor p75. The other half are predominantly GDNF-responsive neurons that express the receptor c-Ret[65] as well as a series of other markers. These markers include the ability to bind a specific Griffonia lectin for fucosyl moieties, isolectin B4, which provides a convenient, but not completely specific, marker for the GDNF-dependent population. Finally, most nociceptors bear the receptor for the chili pepper toxin capsaicin. This receptor, vanilloid receptor type 1 (VR1), is the basis for the burning pain elicited by topical application of capsaicin. It normally responds to heat and to protons; mice genetically engineered to lack VR1 fail to respond to heat and acid.[10] A related receptor, vanilloid receptor–like receptor type 1, has recently been identified, primarily on somewhat larger sensory neurons probably giving rise to Aδ fibers. Vanilloid receptor–like receptor type 1 responds to very high temperatures (>52° C).[11] In the human, almost all of the epidermal axons are VR1 positive.[82] Epidermal fibers containing the peptides calcitonin gene–related peptide (CGRP) and substance P (SP) are NGF-dependent axons. In some animals these commonly enter the epidermis, but in human skin they usually terminate near capillary loops in the dermal papilla. Attempts to immunostain for other peptides in human nerves, including the isolectin B4, have been unsuccessful.

METHODS OF CUTANEOUS NERVE ANALYSIS

Choice of Biopsy/Blister Location

The minimal invasiveness and negligible scarring remove most cosmetic objections to skin biopsy and skin blister procedures. When choosing a biopsy site, the susceptibility to nerve loss with neuropathy and the availability of normative data should be considered. Areas subject to trauma or with scars should be avoided. Site selection also depends upon the purpose for which the biopsy/blister is being considered (e.g., for diagnosis or for longitudinal study). Diagnosis of small fiber pathology can be made from a distal skin location where the clinical examination shows decreased sensation, particularly decreased sensitivity to mechanical (pin) and thermal (hot) pain stimuli. In patients with neuropathy, biopsies from the dorsum of the foot, distal calf, and proximal calf frequently contain a reduced number of epidermal nerves or may be devoid of epidermal nerve fibers altogether. The foot has the advantage of being more distal, but its nerves are subject to trauma. Supplementary biopsies at more proximal levels (e.g., calf, thigh, or upper extremity) can yield additional information about the severity and distribution of small fiber loss (Fig. 34–3). If the plan is to follow the course of neuropathy or the response to therapy, it is best to select a biopsy location with a definite reduction of epidermal nerves, but with a sufficient nerve residual in the epidermis and subepidermal neural plexus to either provide a substrate for nerve regeneration or allow documentation of further degeneration. This can usually be accomplished by removing biopsies from the upper sensory level, if present, and from one or two more proximal locations, keeping in mind that the loss of epidermal nerves is generally more severe than would be inferred from the clinical symptoms or findings. Future biopsies for comparison should be performed horizontally adjacent to the original biopsy at least 5 to 10 mm away from scarring and within the same peripheral nerve distribution. Normal epidermal nerve fiber values have been determined for proximal and distal anterolateral thigh, proximal and distal posterolateral calf, dorsum of the foot over the extensor digitorum brevis muscle, over the first dorsum interosseus muscle, proximal volar forearm, and the paraspinal area at T4-T5. The most frequently biopsied locations selected to assess a suspected length-dependent neuropathy are thigh and calf. The flexibility of the technique allows biopsy or blister to be performed safely almost anywhere on the body. Thus a localized sensory complaint (e.g., a thoracic radiculopathy) can be examined by biopsy of the symptomatic site and a mirror-image control, with postherpetic neuralgia being a possible exception because there may be contralateral changes in the unaffected side.[76]

Biopsy Methods

Skin biopsy is commonly performed with a 3-mm diameter punch instrument (Fig. 34–4) (Acupunch; Acuderm, Inc., Ft. Lauderdale, FL) with local anesthetic. The wound rarely requires cautery or suture, and the infection rate in the lower extremity is less than 1:200. A shallow biopsy is adequate if interest is limited to epidermal nerves. A deeper biopsy (3 to 4 mm) is necessary to acquire sweat glands, the full hair follicle, subcutaneous fat, and larger arterioles. The biopsy is immediately placed into 4° C fixative and stored overnight, then transferred to 20% sucrose in phosphate-buffered saline for cryoprotection and storage. The biopsy can be held in sucrose in phosphate-buffered saline at 4° C for up to 1 month or frozen for more prolonged storage. Formaldehyde-based fixatives provide the best tissue and antigen preservation. Glutaraldehyde destroys the antigens and should not be used. Zamboni (2% paraformaldehyde and picric acid), Lana (4% formaldehyde and picric acid),

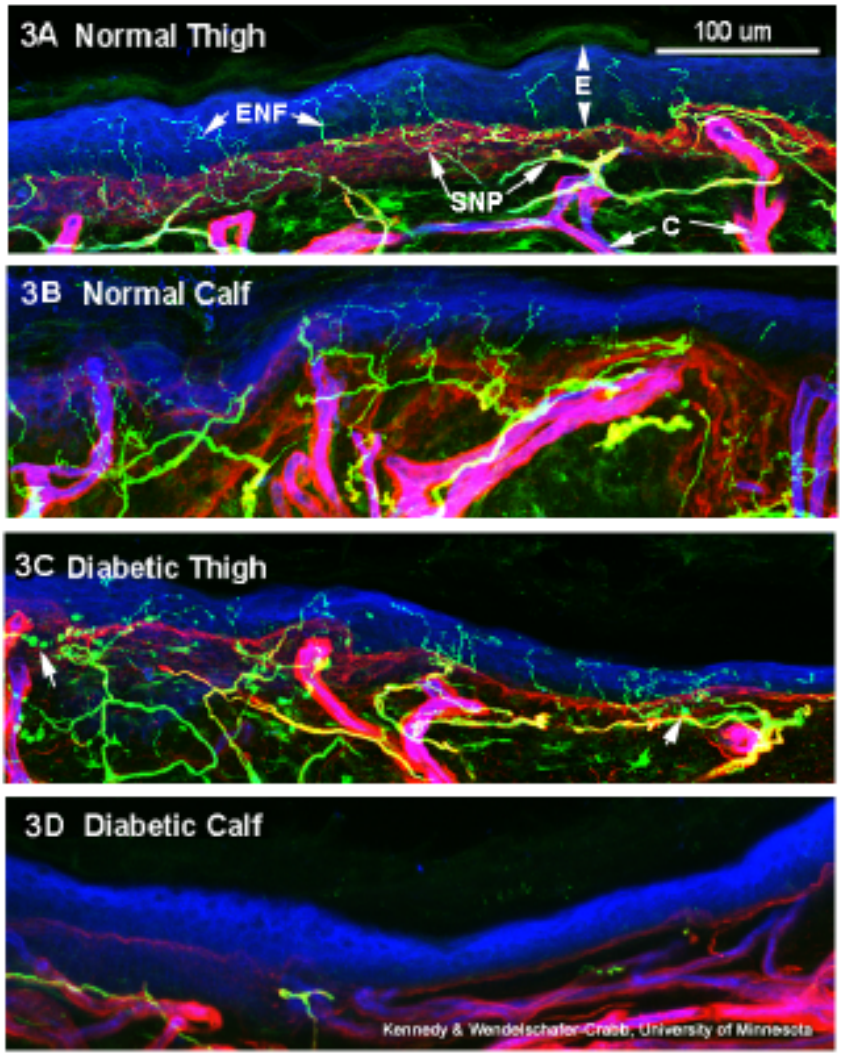

FIGURE 34–3
Cutaneous innervation of normal and diabetic subjects. Confocal microscope images of sectioned superficial skin of a normal (**A** and **B**) and a diabetic (**C** and **D**) subject. Epidermal nerve fibers arise from a subepidermal neural plexus (SNP) that extends horizontally immediately below the dermal-epidermal basement membrane. Numerous nerve fibers cross the basement membrane to innervate the epidermis of a control subject (**A,** thigh; **B,** calf). Diabetic subjects display a range of epidermal nerve fiber density depending on the body location and the degree of neuropathy. Skin biopsies from a diabetic subject have normal density of innervation in the thigh (**C**); however, several abnormalities are observed. Swellings in the nerve fibers are common in the SNP, and some nerve fibers are longer and more branched. The bulbous nerve endings are not seen at other depths of the dermis. The calf biopsy of the same subject contains no epidermal nerve fibers (**D**). A single nerve fiber in the SNP branches at the dermal-epidermal junction but does not cross into the epidermis. (From Kennedy, W. R., Wendelschafer-Crabb, G., and Walk, D.: Use of skin biopsy and skin blister in neurological practice. J. Clin. Neuromuscul. Dis. *1*:196, 2000, with permission.) *See Color Plate*

and PLP (paraformaldehyde, lysine, and periodate) fixatives all provide optimal preservation. If these fixatives are not available, 10% formalin preservation for 12 to 18 hours is an alternative, but produces a more fragmented appearance of the epidermal nerves and is suboptimal. For multicenter studies or trials, in which the biopsy will be performed at a location geographically removed from the processing laboratory, the specimen should be placed into fresh cold fixative for 12 to 24 hours, then transferred into cryoprotectant for overnight shipping on wet ice.

Staining Skin Biopsy Specimens

Nerve evaluation from skin biopsy relies on immunohistochemical localization of neural antigens within skin tissue sections. This is accomplished by applying a primary antibody directed against the antigen of interest followed by a labeled secondary antibody directed to the primary antibody. The major primary antibody used for the localization of nerves in skin biopsy recognizes the pan-neuronal marker PGP 9.5 (Ultraclone, Wellow, UK; Chemicon, Temecula, CA) that is present in all cutaneous nerves. Localization of additional antigens can aid in diagnosis but may not be necessary in all situations. Several antigens can be localized within a single tissue section by using secondary antibodies that recognize and react with the immunoglobulin G (IgG) of the primary antibodies. Typically rabbit, mouse, and goat primary antibodies are localized with donkey antiserum specifically prepared to recognize IgG from only a single species (rabbit, mouse, or goat). Complementary markers such as fluorophores or enzymes label each of the species-specific secondary antibodies. Each fluorophore's emission wavelength peak must be distinct so that it can be viewed individually with appropriate light filters (Fig. 34–5). Alternatively, enzymes such as peroxidase or alkaline phosphatase are reacted with a variety of available substrates to provide reaction products of different colors. All secondary antibodies should be derived from the same species

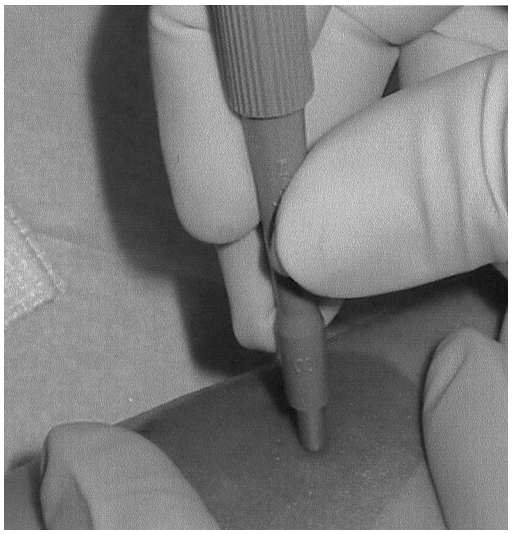

FIGURE 34–4
Punch biopsy tool. A 3-mm punch biopsy is made on clean, shaved skin that has been swabbed with povidone-iodine (Betadine) and numbed with local anesthetic. The biopsy tool is inserted with a twisting motion to approximately half the depth of the metal collar. The depth of the biopsy depends on the thickness of the skin and the sample content desired. Sweat glands lie deep in the dermis and require a full-thickness biopsy, while shallow biopsies are sufficient for analysis of epidermal nerve density.

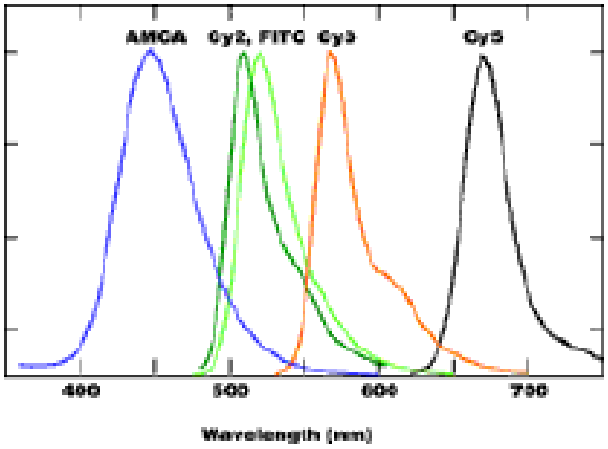

FIGURE 34–5
Fluorophore emission wavelength profile. Fluorophores with distinct wavelengths that can be isolated visually by use of optical filters are used to label antibodies for multiantigen immunofluorescent staining applications. Nerves are usually labeled with Cy 3 (fluorescence peaking at 565 nm), while basement membrane is labeled with Cy 2, fluorescing at 520 nm. By applying a blocking filter between 540 and 550 nm, the two antigens can be viewed independently; while applying a dual-band filter, the two antigens can be viewed simultaneously and distinguished microscopically by their colors. (Courtesy of Jackson ImmunoResearch, West Grove, PA.)

(e.g., donkey) and must be from a species different than hosts of the primary antibodies. Secondary antibodies used for multiple staining must be preadsorbed with IgG from all species other than the primary antibody being localized.

Frozen 50- to 100-μm-thick sections are cut perpendicular to the epidermal surface. The thicker sections provide greater sampling and visualization of anisotropic nerve fibers as they weave through the skin. Sections are floated in staining reagents containing Triton X-100 to promote penetration of antibodies. Samples are washed and antibodies are diluted in a solution of phosphate-buffered saline, 1% normal donkey serum, and 0.3% Triton X-100. Sections are incubated in 5% normal donkey serum so that nonspecific binding of IgG will be "blocked." Sections are incubated in primary antibodies (e.g., rabbit anti–PGP 9.5 and mouse anti–type IV collagen; Chemicon) with gentle rotation for 5 hours at room temperature and overnight at 4° C, then washed in three changes of wash buffer, 1 hour each, with gentle rotation. This is followed with overnight incubation in labeled secondary antibody to the rabbit and mouse primary antibodies (Jackson ImmunoResearch, West Grove, PA), then washed three times. The secondary antibodies are labeled with either peroxidase or a stable fluorescent marker.

Peroxidase markers are reacted enzymatically to produce a precipitate at the site of the antigen. The peroxidase reaction product is visible by bright-field microscopy and by electron microscopy. With the peroxidase reaction, the basement membrane is localized as being just below the basal membrane of basal keratinocytes. Fluorescent markers require no further reaction before being studied by fluorescence or confocal microscopy. A new generation of fluorescent markers, Cy dyes 2, 3, and 5 (Jackson ImmunoResearch), are very bright and remain stable for several years in permanent mountants. The markers are useful for labeling two or three antigens within the same section. Labeling nerve with Cy 3, basement membrane with Cy 2, and endothelial cells with *Ulex europaeus* agglutinin type I with Cy 5 provides a vivid image of skin structure, innervation, and vasculature (Fig. 34–6).

Non–immune serum specificity controls are essential and should be run with each biopsy. Accurate counts of epidermal nerve fibers, interpretation, and quantitation are difficult when sections fold or curl during drying of the epidermis. For optimal viewing and quantification, the epidermis should lie flat on the slide. Premounting sections in a small drop of Nobel agar prevents drying, adheres the sections, and holds the surface of the stratum corneum perpendicular to the slide. The premounted section is dehydrated in alcohol, cleared with methyl salicylate, and mounted on a coverslip in DPX (Fluka, Buchs, Switzerland). This provides a permanent preparation and is useful for slides labeled with Cy fluorophores and some enzyme reactions. Glycerol can be used to coverslip tissue without dehydrating the specimens and is useful for short-term storage.

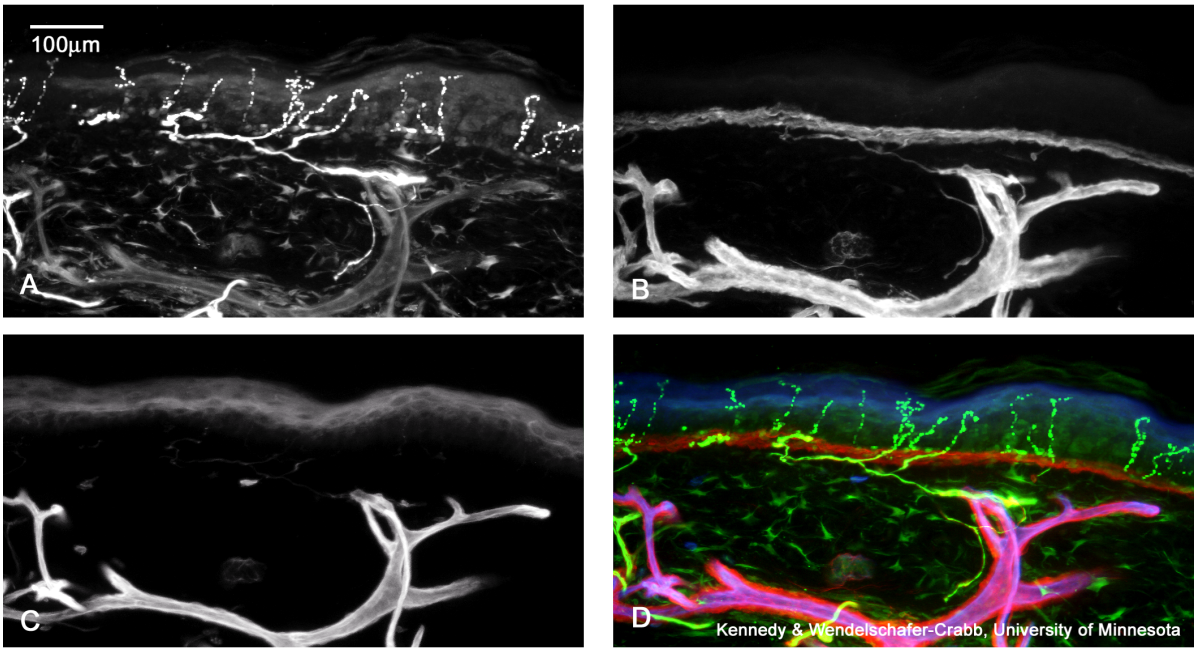

FIGURE 34–6
Staining nerve and basement membrane. **A,** Nerves are immunostained with rabbit anti–PGP 9.5 and Cy 3. **B,** Basement membrane is immunostained with mouse anti–type IV collagen and Cy 2. **C,** Epidermis and vessels are stained with the lectin *Ulex europaeus* agglutinin type I and Cy 5. **D,** All images are combined in this colorized representation of the localization of nerves, vessels, dermal-epidermal basement membrane, and epidermis. Confocal images are acquired as gray-scale image stacks for each antigen-fluorophore combination. These are projected, pseudo colored, and combined to create a color image of the entire z series. *See Color Plate*

Skin Blister Method

Skin blister is a painless, bloodless method to acquire, quantify, and plot distribution of epidermal nerve fibers.[43] The blister roof, which consists of pure epidermis and epidermal nerve fibers, separates from the dermis on a plane between the basal membrane of basal keratinocytes and the dermal-epidermal basement membrane. Blisters are made by applying suction to a blister capsule (WR Medical Electronics, Stillwater, MN) that is adhered to shaved, cleansed skin with double-sided tape (Fig. 34–7). The surface of the capsule resting on skin contains one or more round openings to accommodate the desired number and size of blisters. Tegaderm tape (3M, Maplewood, MN) disks, cut slightly smaller than the size of the desired blister (e.g., 3-mm diameter), are placed on the skin surface to conform to the openings in the blister capsule. These prevent overstretch of the blister roof. The capsule is secured to skin with an elastic bandage, leaving the transparent top of the capsule uncovered to permit the examiner and subject to observe blister formation, and the capsule is evacuated to a negative pressure of 300 mm Hg. The small volume of the capsule makes it imperative to have tight seals to prevent leakage. A reservoir placed in series with the tubing between the pump and capsule enhances blister formation by equilibrating small leaks. We use a commercially available mechanical pump to evacuate the capsule, but an inexpensive hand-held pump is adequate when a single capsule is used. Initially small blisters form, then these coalesce to form a full blister after 20 to 40 minutes. The time is less with increasing subject age and skin temperature, but also depends upon skin location and successful maintenance of negative pressure. Warming the area

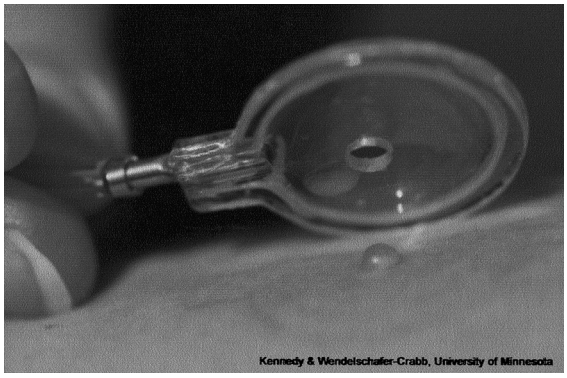

FIGURE 34–7
A blister formed by application of negative pressure in the blister capsule. The blister conforms to the 3-mm opening in the base of the capsule. (From Kennedy, W. R., Nolano, M., Wendelschafer-Crabb, G., et al.: A skin blister method to study epidermal nerves in peripheral nerve disease. Muscle Nerve 22:360, 1999, with permission.)

with a heating pad hastens blistering. After the capsule is removed, the blister roof with tape disk intact is excised with microscissors and placed in cold Zamboni fixative overnight at 4° C. Fixed blisters are cryoprotected with 20% sucrose in 0.1 M phosphate-buffered saline until processed. Prior to processing, blister roofs are frozen on dry ice. Specimens are processed as whole mounts without sectioning; otherwise the procedure is identical to that for thick sections.

Epidermal Sheet Preparations

This is a modification of the standard punch technique that allows for separation of the epidermis from the dermis before fixation.[5] The technique is particularly useful for examining the epidermis in the horizontal plane without dermal vessels or nerves. In addition, the epidermal sheet can be used for protein or messenger RNA (mRNA) assays, as described below, rather than immunocytochemical staining. Briefly, after obtaining the punch biopsy, the specimen is bathed in ethylenediaminetetraacetic acid for 2 hours; then the epidermis can be easily removed from the dermis, fixed, and immunostained as above. This preparation permits the identification of the nerve fiber terminals within the epidermis without blood vessels or the dermal structures. The sheet can be stained in exactly the same way to identify PGP-immunoreactive nerve fibers.

Polymerase Chain Reaction Techniques in Skin

It is feasible to quantify neurotransmitter or neurotrophin mRNA levels in either the epidermis (using the sheet preparation) or the dermis (using the standard punch biopsy). In the field of HIV/acquired immunodeficiency syndrome (AIDS), there is interest in the concept that specific antiretrovirals may affect mitochondrial DNA levels by inhibition of gamma DNA polymerase.[13] Subcutaneous fat is a rich source of mitochondria. We use the subcutaneous fat, which is usually trimmed and discarded from punch skin biopsies, to measure levels of mitochondrial DNA with real-time polymerase chain reaction[23] in HIV-seropositive patients receiving antiretrovirals. Specific antiretrovirals, the dideoxynucleosides, reduce mitochondrial DNA levels by inhibition of gamma DNA polymerase,[60] and thus this technique may provide a convenient measure of the severity of dideoxynucleoside toxicity and the metabolic complications common in individuals treated with these drugs long term.[14]

MICROSCOPY

Thick sections are advantageous for viewing the full meandering course of nerves from the nerve trunk in the dermis throughout their ramifications to the endings in sweat glands, arrector pili, hair follicles, arterioles, or epidermis. Sections are first scanned under a 10 × objective in the microscope to evaluate the orientation and flatness of the section and staining of nerves, blood vessels, and cutaneous organs. The course and ending of single unmyelinated nerve fibers are better visualized with a 20 × (or higher) objective, as, for example, when following epidermal nerve fibers through basement membrane and the pathway between keratinocytes to their endings under stratum corneum. Images taken from thick sections are often marred by out-of-focus blur because the focal plane of a 20 × objective is about 2 μm. Objects in 50- to 100-μm sections that are above and below this focal plane are out of focus. Some laboratories use thinner sections to obtain sharp images by conventional microscopy. This is counterbalanced by the inability to follow nerves for more than a short distance in a section and reduced sampling.

Confocal microscopy of thick sections is one solution to this problem, although the added complexity may not always be necessary for simple clinical questions. The confocal microscope takes in-focus images while retaining the advantages of working with thick sections. This is accomplished by "optically sectioning" the fluorescent-labeled thick sections into a series of smaller increments that correspond to the focal length of the lens being used. A confocal z series comprising multiple images taken at incremental focal planes throughout the tissue eliminates out-of-focus blur and provides an in-focus three-dimensional view of the tissue (Fig. 34–8). The operator can select the top and bottom of the section to be imaged, the size of the z-focus increments, and the fluorophores to image before collecting the z series. A laser scanning confocal microscope requires about 30 minutes to scan double-stained sections (two antibodies) at 2-μm increments through 50 μm of tissue. The nonlaser spinning disk confocal system (e.g., CARV) can collect equivalent images in about 3 minutes. The z series is projected into a single in-focus image for viewing. The series can also be "paged through" to follow nerves throughout the tissue, or the series can be rendered into a three-dimensional object.

QUANTITATION OF CUTANEOUS NERVES

Epidermal Nerves

Standard punch biopsies removed from anatomic locations that are often sampled to diagnose neuropathy contain an adequate sample of unmyelinated epidermal nerves and subepidermal neural plexus for detecting neuropathic changes. Epidermal innervation can be readily quantified using either confocal microscopy[46,78] or direct counting of chromogen-stained specimens.[16,32] The diagnosis of neuropathy is dependent upon finding

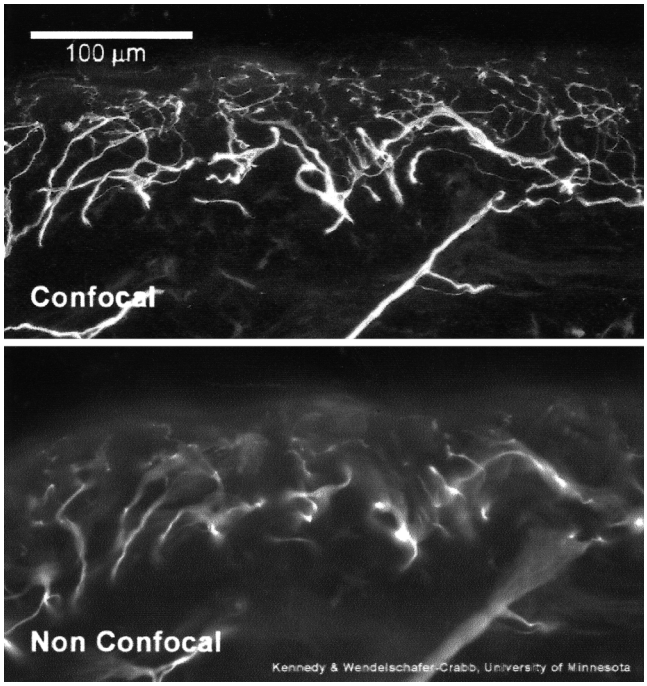

FIGURE 34–8
Confocal versus nonconfocal microscopy. The confocal microscope uses focused light beams (laser or mercury vapor) to optically section thick tissue sections, much like a computed tomography scanner. A series of many images are acquired at focal increments specific to the objective lens' depth of field. Out-of-focus blur is eliminated, and clear, in-focus images of sections up to 200 μm thick can be collected and used for three-dimensional analysis of nerve morphology and quantification.

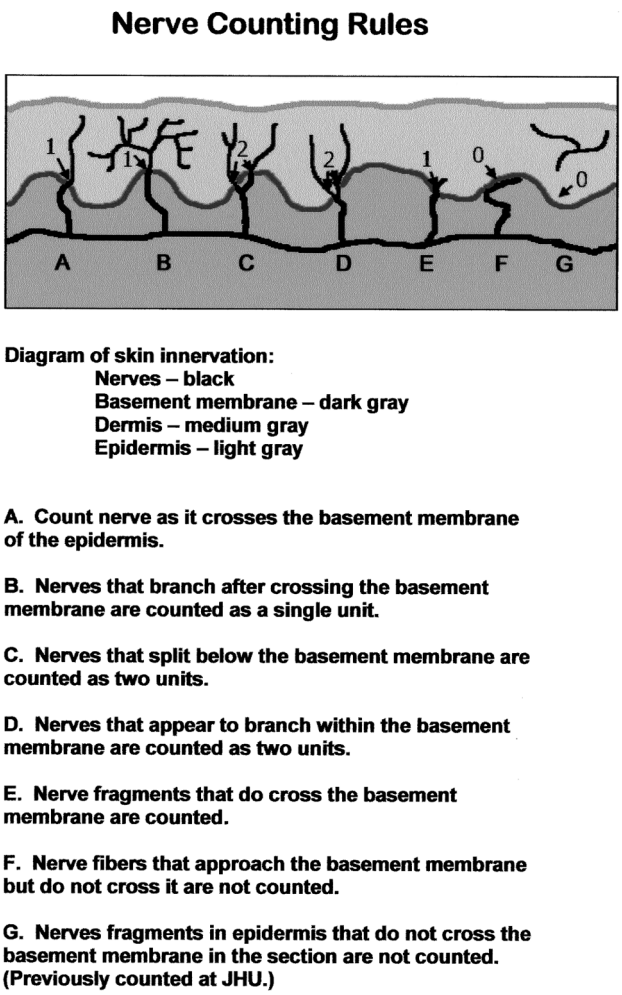

FIGURE 34–9
Nerve counting rules. A set of concise rules was developed to normalize counting among investigators.

a reduced density of epidermal nerves. Abnormalities of nerve morphology provide valuable secondary help. Epidermal nerve fibers can be quantified accurately because, after they leave the nerve bundles of the subepidermal nerve plexus and before they enter the epidermis, they separate as single nerve fibers. In thick sections the entire intraepidermal segment of most epidermal nerve fibers can be followed rather than isolated nerve segments, as is necessary in thinner sections. A set of almost identical counting rules evolved simultaneously in laboratories at both the University of Minnesota and Johns Hopkins University (Fig. 34–9):

A. *Count each nerve as it crosses the basement membrane of the epidermis*. Because epidermal nerve fibers often branch below the basement membrane as well as in the epidermis, it is necessary to establish a standard counting site. Most investigators have agreed in practice to count as one unit each nerve that penetrates the basement membrane, even if there is branching within the epidermis. In peroxidase-stained sections, the membrane and cytoplasm of basal keratinocytes stain darker than surrounding tissue. Because the basal membrane is only a few micrometers above the dermal-epidermal basement membrane, it can be used as the site for counting penetrating epidermal nerve fibers. In sections that are double-stained for PGP 9.5 in nerve and type IV or type VII collagen for basement membrane and labeled with compounds that fluoresce at different wavelengths, it is possible to directly observe the epidermal nerve fibers as they penetrate basement membrane. If confocal images of thick sections are available, the optical section in which the epidermal nerve fiber penetrates the basement membrane is located by paging through the z series of optical sections.
B. *Nerves that branch after crossing the basement membrane are counted as one*. Epidermal nerve endings in normal subjects are usually quite simple—often free of branches or with minimal branching as they course through the epidermis. Nerves of patients with neuropathy frequently appear to have increased fiber

branching. To address both nerve loss and collateralization of surviving axons, the number of fibers crossing the basement membrane is used to provide counts (how many nerves) while analysis of tracing data provides information about the complexity (length and branching pattern) of the nerve (how much nerve).

C. *Nerves that split below the basement membrane are counted as two units*. Single epidermal nerve fibers arise as branches from nerve bundles of the subepidermal neural plexus. Each branch is counted as it penetrates the basement membrane.
D. *Nerves that appear to branch at the basement membrane are counted as two or more units*.
E. *Nerve fragments that cross the basement membrane are counted*. Epidermal nerve fibers that appear short are counted. They may be short fibers or the roots of fragments that terminate in adjacent sections.
F. *Nerve fibers that approach but do not cross the basement membrane are not counted*. Nerve fibers that terminate immediately proximal to the basement membrane are common in neuropathy. They frequently follow the basement membrane a short distance before ending with a terminal swelling.
G. *Epidermal nerve fragments that do not cross the basement membrane are not counted*. This rule has been followed in all publications by the Minnesota group. In previous publications by the Johns Hopkins group, such epidermal nerve fragment were included in the total numbers, but they will not be included in future counts.

Tracings made of epidermal nerve fibers with a software program (Neurolucida; MicroBrightField, Williston, VT) provide the number of nerve fibers, branch points, nerve fiber length, and coordinates of basement membrane penetration (Fig. 34–10). This information is useful because in some patients nerve branching and length increase in apparent compensation for a decrease in nerve number. The data are also useful for analysis of nerve distribution.

Results of epidermal nerve fiber density are commonly expressed as number of epidermal nerve fibers per millimeter of epidermis, providing a "linear density." Comparison of such values from different reporting laboratories requires that the thickness of the section be known because thicker sections contain more nerve fibers. Results can also be reported as epidermal nerve fibers per square millimeter area of skin surface for easier comparison.[32,46] Measurement accuracy is potentially adversely influenced by compression of the section between the slide and coverslip and by tissue shrinkage during preparation. For accurate comparison, these factors must be taken into consideration. Our laboratories report epidermal nerve fiber density based on nerve counts made in the equivalent of 50-μm-thick sectioned tissue, making adjustments for changes resulting from dehydration and coverslip compression.

Manual counting should be performed on at least three to four nonadjacent sections taken from different places within the biopsy. After manually counting the number of individual epidermal nerve fibers, the length of the epidermis is measured using Bioquant V (R&M Biometrics, Nashville, TN). Counting rules should be established a priori for each laboratory, and preferably only one observer used for research studies involving serial specimens. We have compared manual counting with light microscopy to stereologic techniques and demonstrated that the manual counting produced comparable densities but was much quicker.[101] Reliability is highest when at least four sections at each anatomic site are counted. A technician can be trained to perform the manual quantitation, with excellent inter- and intraobserver reliability (>0.95).[61] We have also demonstrated that different laboratories can achieve reasonable interlaboratory reliability.[98] For example, mean (± standard error of the mean) interobserver reliability was 12.2% ± 1.1% for each biopsy site and mean intraobserver variability was 10.2% ± 1.5% for individual sections. The variability between laboratories was 25.5% and the correlation coefficient for both intraobserver and

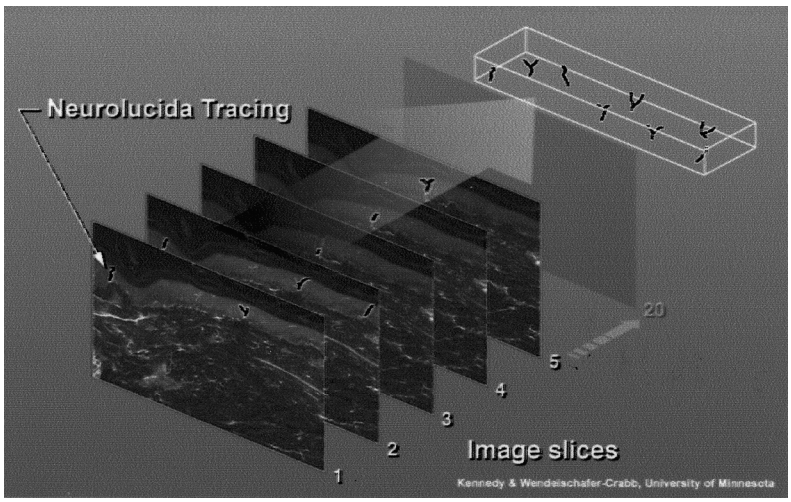

FIGURE 34–10
Nerve quantification by tracing. Neurolucida software (MicroBrightField, Williston, VT) uses the confocal image series to identify and trace each nerve fiber from the point where it crosses the basement membrane to its termination within the epidermis. Nerve number, length, complexity, and density as well as epidermal length and volume can be determined from the acquired data.

interobserver reliability was .98. The correlation coefficient between sites was .94. There was no relationship between absolute intraepidermal nerve fiber counts and reliability.

Normal Epidermal Nerve Density

The density of epidermal nerves varies depending upon the biopsy site.[40] In general, a consistent gradient in intraepidermal nerve fiber density exists from proximal to distal sites in the lower extremities, with minimal effects of race or sex. Significantly higher intraepidermal nerve fiber densities were noted in a younger group, age 10 to 19 years, but over the age of 20 years there is little change up to 80 years of age (Fig. 34–11).[15,78] Stereologic quantitation has been compared with linear density estimation and is significantly associated with a correlation coefficient of .79 ($P = .001$). With formalin fixation (which tends to underestimate by about 20% compared to PLP fixation), the normative range for intraepidermal nerve fiber density (including intraepidermal fragments) in healthy normal controls is 21.1 ± 10.4 fibers/mm (mean and standard deviation) in the thigh, with a fifth percentile of 5.2 fibers/mm. At the distal leg the normative range is 13.8 ± 6.7, with a fifth percentile reading of 3.8 fibers/mm. Using the cutoff values for the fifth percentile for intraepidermal nerve fiber density at the distal part of the leg, the diagnostic efficiency (percentage correctly classified) was 88%, with a specificity (percentage true negative) of 97% and a sensitivity (percentage true positive) of 45%. Positive predictive value was 92% and negative predictive value was 90%. Although there is a relatively wide biologic variation in the intraepidermal nerve fiber density, the distribution of densities at the distal part of the leg permits the delineation of lower limit of normal based on the fifth percentile value for healthy controls. The high specificity of the measure increases the positive predictive value of the test, which is therefore ideal for its anticipated use, namely to verify the presence of a disease for which there may be little clinical or electrophysiologic evidence, or when the clinician must be virtually certain of a diagnosis, for example, before initiating some form of therapy. The relatively low sensitivity of the measure implies that a normal intraepidermal nerve fiber density does not rule out the presence of a sensory neuropathy. Normative values of epidermal nerve fiber density for six body locations have been determined at the Kennedy laboratory (Table 34–1).

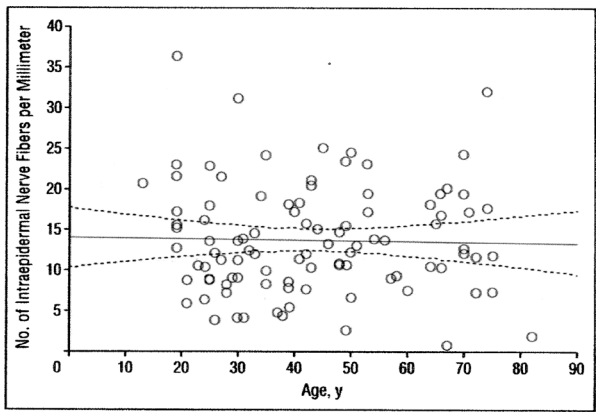

FIGURE 34–11
Effects of age on intraepidermal nerve fiber density at the distal part of the leg by age decile for healthy controls. (From McArthur, J. C., Stocks, E. A., Hauer, P., et al.: Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch. Neurol. 55:1513, 1998, with permission.)

Epidermal Nerves in Skin Blisters and Epidermal Sheets

Whole-mounted blister and epidermal sheet preparations have sampling advantages over sectioned skin biopsies. The "bird's-eye" perspective of epidermal nerve fibers in these 3-mm diameter (area = 7.1 mm^2) preparations presents for visualization and analysis the same number of nerves contained in the combined sections of a 3-mm biopsy (Fig. 34–12). Using a $20 \times$ objective lens, we count the epidermal nerve fibers in five representative segments of the blister roof, each measuring 0.4×0.3 mm. The 0.6-mm^2 skin surface area is approximately 12% of the whole blister roof. The results, expressed as counts of epidermal nerve fibers per surface area of skin, vary according to body location in the same manner as that observed with biopsies. Epidermal nerve fiber density determinations from biopsies and blisters show a high degree of correlation ($r = .71$; $P < .005$).[49] The blister roof also provides an advantageous oversight of the horizontal territory of individual epidermal nerve fibers and the distribution of all epidermal nerve fibers within the specimen. Changes in epidermal nerve distribution caused by reorientation of nerves in their course through the epidermis by elongation, branching, or loss of branching probably occur normally during the continual regeneration of the epidermis, perhaps similar to normal reshaping of the terminals of motor axons.[57] We suspect that exaggerated redistribution of epidermal nerves may be the earliest reaction of epidermal nerves to neuropathy (L. Waller, personal observations, 1998).

Other Cutaneous Nerves

In general, dermal nerves are more difficult to quantify than epidermal nerve fibers. The subepidermal neural plexus that occupies the papillary dermis is often less dense in subjects with neuropathy. These bundled nerve fibers cannot be accurately counted or traced. The volume of nerve in the subepidermal neural plexus has been

Table 34–1. Epidermal Nerve Fiber Density*

Normal	Foot	Calf	Thigh	Hand	Forearm	Back
Mean	22.83	18.58	34.99	24.57	37.57	62.61
95% Cutoff	12.80	6.73	19.60	10.40	17.10	33.00
Standard Deviation	7.61	6.54	15.88	11.31	12.96	21.25
Number	39	39	39	38	37	23

*Normative data have been determined at the University of Minnesota for proximal and distal anterolateral thigh, proximal and distal posterolateral calf, dorsum of the foot over the extensor digitorum brevis muscle, over the first dorsum interosseus muscle of the hand, the proximal volar forearm, and the paraspinal area of the back at T4 to T5.

estimated based on the total fluorescence,[55] but accurate quantification of nerves in the subepidermal neural plexus by thresholding from background is complicated by the positive immunoreactivity of dermal fibroblasts for PGP 9.5. A semiquantitative rating system can be used to assess the density of the plexus (Fig. 34–13): +1 = hyperinnervation, 0 = normal, −1 = mild (50%) loss, −2 = severe (75%) loss, −3 = a trace, and −4 = no nerve. A similar semiquantitative rating system can be used to quantify sudomotor nerves. These rating systems require that the

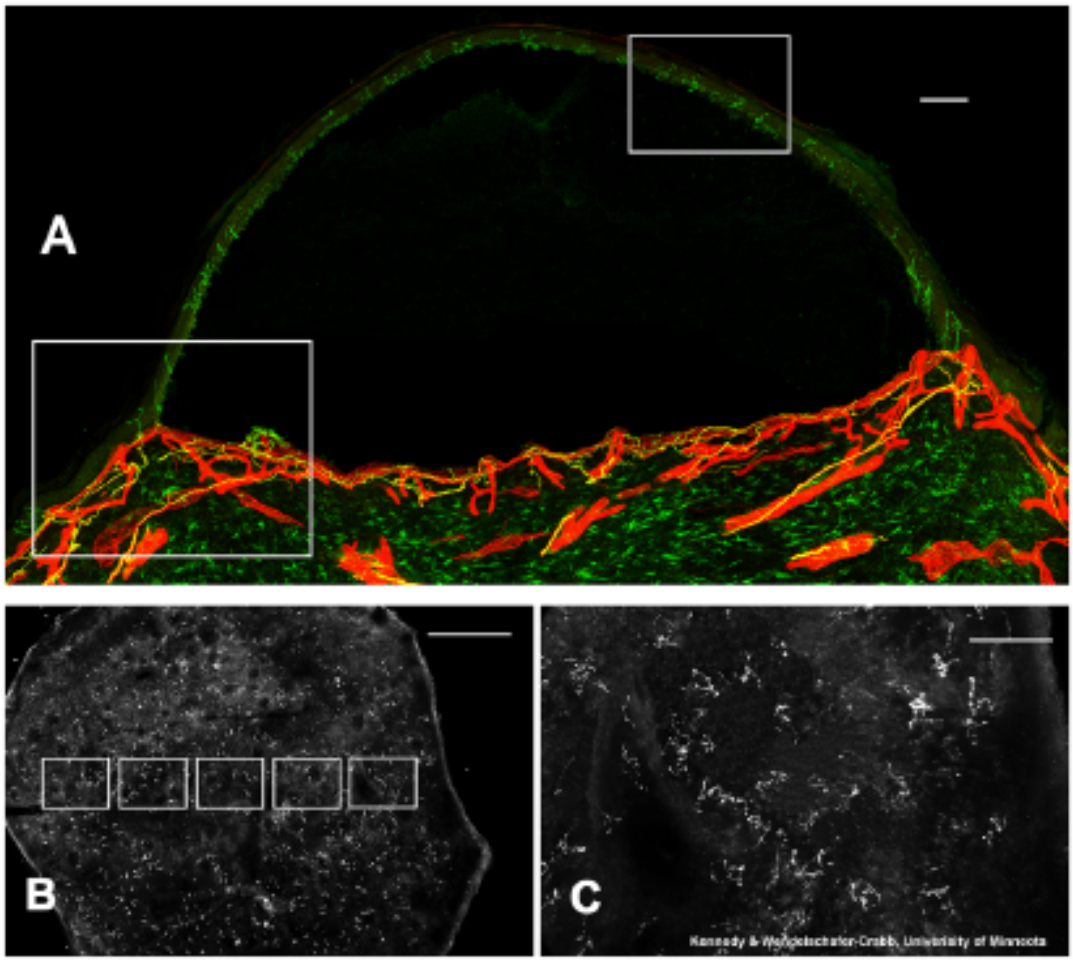

FIGURE 34–12
Blister analysis. **A,** Blister profile: confocal image of an immunostained sectioned blister. Nerves (PGP 9.5) are green or yellow, and basement membrane (type IV collagen) is red. Epidermis separated from dermis just above the dermal-epidermal basement membrane. Dermal capillaries and the subepidermal neural plexus remained intact (*left box*). Epidermal nerves, severed from their proximal segment, remained in the blister roof (*right box*). Bar: 100 μm. **B,** Survey confocal image of a 3-mm blister roof immunostained for nerve with antibody to PGP 9.5. Rectangles indicate the areas imaged at 20 × for nerve counts. Bar: 500 μm. **C,** Higher magnification image is used for quantification of epidermal nerve fibers (from right rectangle in **B**). Bar: 100 μm. (From Kennedy, W. R., Nolano, M., Wendelschafer-Crabb, G., et al.: A skin blister method to study epidermal nerves in peripheral nerve disease. Muscle Nerve 22:360, 1999, with permission.) *See Color Plate*

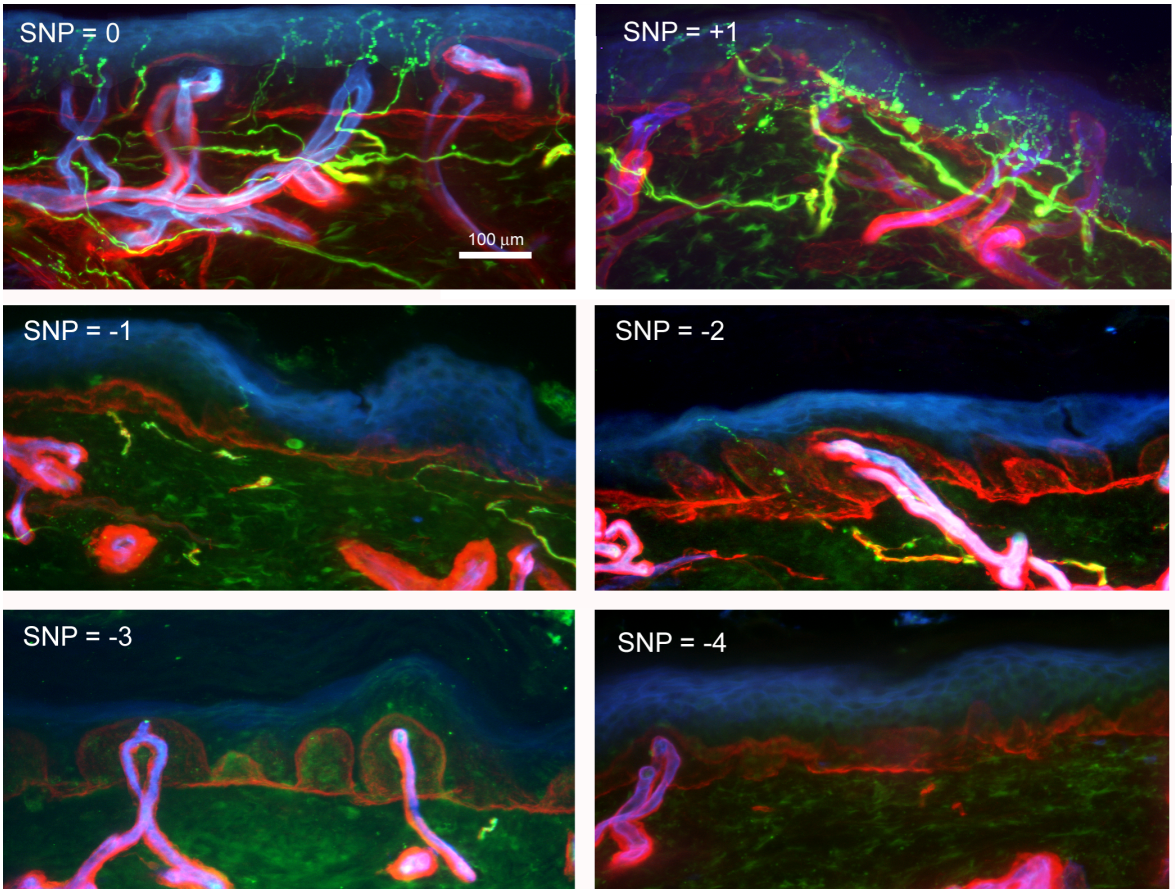

FIGURE 34–13
Subepidermal neural plexus (SNP) rating. A semiquantitative rating system can be used to assess the density of the plexus. SNP = 0 indicates a normal innervation pattern in the papillary dermis with nerve bundles running subjacent and parallel to the epidermis. SNP = +1 denotes hyperinnervation, a condition that is encountered in early stages of neuropathy when active regeneration is occurring. It is often associated with morphologic abnormalities such as swellings, clustering of nerves, and truncation of nerve fibers at the dermal-epidermal basement membrane. SNP = −1 signifies a moderate (50%) loss of SNP innervation, and is usually accompanied by a reduction in epidermal nerve fiber density. SNP = −2 indicates a severe (75%) loss. SNP = −3 applies when only a trace of SNP remains. SNP = −4 indicates that no nerve remains in the papillary dermis. *See Color Plate*

viewer have an excellent interpretation of "normal." Other techniques utilizing image analysis to quantify cutaneous nerves have recently appeared[107] but have not yet been widely accepted.

The density of sudomotor nerves around secretory tubules of sweat glands can be quantified by calculating nerve volume per sweat gland volume in confocal images.[46] Sudomotor nerve density is below normal in patients with severe neuropathy (Fig. 34–14), whereas sweat glands often appear well innervated in mild or moderate neuropathy, even though the secretion of sweat is decreased. This suggests that quantitation of sudomotor nerves may not be helpful for early diagnosis of diabetic neuropathy. A possible explanation for this apparent discrepancy is that sweat glands in nonhairy human and murine skin receive multiple sudomotor nerves. Multiple innervation facilitates collateral reinnervation after a partial nerve lesion,[44] but sweat volume may be compromised. The occasional findings of increased sweat gland innervation[46] and of increased sweat secretion[58] in proximal skin locations of diabetic subjects may represent localized nerve growth in response to a general stimulus for collateral reinnervation during the early denervation stage of neuropathy.

We have not attempted to quantify the innervation of arrector pilorum muscles, hair follicles, or arteries. Quantitation of the nerves to arrector pilorum muscles is possible with the quantitative methods described for sweat glands. This may be useful in the clinical diagnosis of peripheral autonomic nerve disease if adequate sampling of these muscles is achieved. Hair follicles have an abundant supply of associated myelinated and unmyelinated nerve fibers, but the relatively few follicles visualized in biopsy of nonhairy skin create a sampling problem. Evaluation for

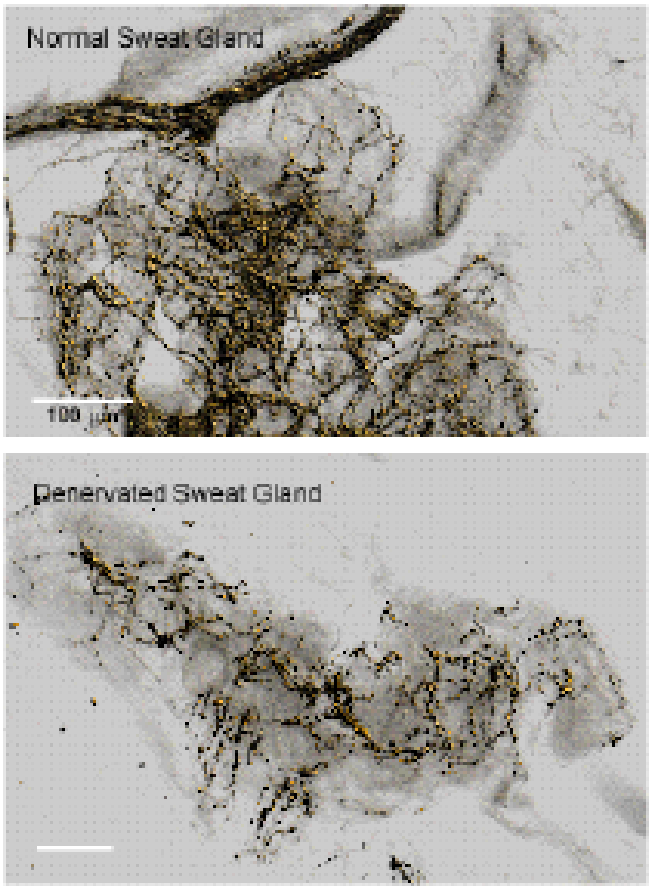

FIGURE 34–14
Sweat gland nerve density. These images depict the innervation of sweat glands from normal (**top**) and diabetic (**bottom**) human skin. Samples used for quantification are immunostained with anti–PGP 9.5 and imaged confocally. Nerve volume is computer-rendered.

clinical purposes may be possible in the scalp, depending upon the number of follicles available for analysis. The wide distribution of cutaneous arteries and arterioles and the variable number of nerves per vessel complicate attempts to quantify vasomotor nerves.

Morphologic Changes in Sensory Neuropathy

In healthy subjects, epidermal fibers arise from subpapillary nerve fiber bundles and run toward the stratum corneum in either branched or unbranched patterns. The fibers are usually thin, are sometimes varicose, and often end with a single clublike enlargement. In cases of sensory neuropathy, intraepidermal nerve fibers show distinct morphologic changes. Fibers frequently show more tortuous courses and more complex ramification.[16,53] In addition to the increased branching complexity, there often is increased varicosity with stubby, rounded projections; intra-axonal swellings; and clawlike terminals. In general, these morphologic features, particularly the intra-axonal swellings, are more evident at the thigh than in regions below the knee (see Fig. 34–3C). The composition of intra-axonal swellings remains uncertain. Neurofilament stains are negative and electron microscopy studies to date have been relatively inconclusive, suggesting that the swellings represent collections of organelles. Because of their location proximal to sites with epidermal denervation, we believe that swellings represent predegenerative changes. In models of epidermal regeneration, clusters of terminal nerve fiber swellings appear more commonly in neuropathic individuals, suggesting that such clusters of swellings may represent aberrant efforts at nerve sprouting, or microneuromas.

The interaction of nerve with basement membrane may be altered in neuropathy as evidenced by morphologic irregularities found in patient biopsies. Some nerve fibers follow a course along the dermal side of the basement membrane but do not penetrate, frequently ending in a bulblike swelling (see Fig. 34–3D). In patient biopsies with low nerve fiber density, nerves entering the epidermis often branch at the point of penetration through the basement membrane and form a tuft of nerve endings that extend into epidermis. Similarly, clustering of epidermal nerve fibers is frequently seen in disease states, wherein multiple nerve fiber penetrations are interspersed with relatively long segments of nerve-free epidermis.

Correlation with Tests of Unmyelinated Nerve Function

Relatively few studies have examined the relationship of epidermal nerve fiber densities to other tests of nerve function.

Heat pain has been examined in humans using a chemical (topical capsaicin) denervation model. While no correlation was found between heat pain sensitivity and epidermal nerve fiber density using a large (900-mm^2) diameter probe, statistically significant correlations between fiber density and heat pain sensitivity were found when a small (7.1-mm^2) probe delivered the stimulus.[50] This apparent incongruity is believed to result from the spatial summation of surviving epidermal nerves plus stimulation of the proximal stumps of degenerated epidermal nerves in the dermis (some of which may be sensitized by the degeneration of the nerve ending) that occurs when the larger probe heats a deeper area. The smaller probe heats a more isolated shallow area, with fewer nerve endings activated.

Comparisons of intraepidermal nerve fiber densities with just noticeable difference (JND) thresholds for cooling and vibration levels were made in HIV-associated sensory neuropathies using the CASE IV instrument (WR Company, Stillwater, MN) and were inversely correlated. Because high JND indicates decreased sensitivity to sensory stimuli, it is not unexpected that the correlation estimates were negative.

A correlation analysis based upon the percentile distribution rather than the JND is another statistical approach, but given that the sample size was relatively small and that many of the subjects had mild neuropathy, this analysis was based on the categorization of subjects as normal/abnormal based on their JNDs. Surprisingly, intraepidermal nerve fiber density correlated inversely more closely with vibratory threshold than with cooling threshold. Vibration is subserved by Aβ fibers that are not present in the epidermis while cool threshold detection is mediated by Aδ fibers, which may be included in measurements of intraepidermal nerve fiber densities. An association with C fibers, which subserve noxious heat and mechanical pain and represent the bulk of epidermal nerve fibers, was not included in the QST battery. The inverse correlation of intraepidermal nerve fiber density and vibratory threshold may be partially explained by concurrent involvement of large and small fiber nerves. Furthermore, the fact that only a minority of subjects (43%) had abnormal epidermal nerve fiber densities at the distal leg at baseline suggests that the severity of the neuropathy in these subjects was relatively mild (and perhaps that the epidermal denervation in these subjects had not progressed above the ankle).[82] In another study of 32 patients with painful, burning feet there was no concordance between the results from quantitative sudomotor axon reflex testing (QSART), a QST (vibration and cooling by CASE IV) performed on the upper and lower extremities, and skin biopsy from the distal calf, in which 28 (87.5%) had a reduction of intraepidermal nerve fibers.[78]

Intraepidermal nerve fiber densities have also been assessed in small fiber and autonomic neuropathies, and compared with autonomic screening tests. In a study that was designed to quantify the severity of autonomic impairment in patients with painful neuropathy, excellent correlation was observed between QSART and cooling abnormalities and loss of intraepidermal nerve fiber density.[73] The QSART quantitatively evaluates the postganglionic sympathetic sudomotor axon[58] (see also Postural Tachycardia Syndrome below).

Correlation between Epidermal Nerve and Sural Nerve

Twenty-six patients with neuropathic complaints had sural nerve morphometry and determination of epidermal nerve fiber density at the distal part of the leg. Nonparametric correlations were used because of the nonlinearity of the values. Intraepidermal nerve fiber density correlated with the densities of total myelinated fibers within the sural nerve ($r = .57$, $P = .0011$), small myelinated fibers ($r = .53$, $P = .029$), and large myelinated fibers ($r = .49$, $P = .0054$). There was a trend toward an association between intraepidermal nerve fiber density and sural nerve unmyelinated nerve fiber densities ($r = .32$, $P = .054$). Sensory nerve action potential amplitudes and large myelinated nerve fiber densities were highly correlated ($r = .87$, $P < .0001$). Intraepidermal nerve fiber density and sural nerve small fiber measures were concordant in 73% of patients. In 23% of cases, reduced intraepidermal nerve fiber density was the only indicator of small fiber depletion. It was usually normal in acquired demyelinating neuropathies and where the clinical suspicion for neuropathy was low. The inferences are that determination of distal leg intraepidermal nerve fiber density may be more sensitive than sural nerve biopsy in identifying small fiber sensory neuropathies and that intraepidermal nerve fiber density and assessments of large nerve fibers by nerve biopsy and electrophysiology are all useful for characterizing sensory and sensorimotor neuropathies.[31]

Utility of Skin Biopsy

It is important in clinical practice to have a good sense of the utility (and limitations) of skin biopsy for evaluation of the peripheral nervous system. The biopsy can only provide information about the most distal terminals of sensory and autonomic nerves where they meet their target organ. Interpretation can be affected by local trauma, other diseases, or biopsy artifacts. Nonetheless, biopsy of proximal and distal sites can provide inbuilt intraindividual controls. We have found the punch skin biopsy technique to be clinically useful for defining presence and severity of focal neuropathy and of distally dominant sensory neuropathy and for differentiation of neuropathy from radiculopathy. It remains to be conclusively demonstrated that skin biopsy can track changes in neuropathies over time, or with regenerative treatments, although the technique has this potential.

CONTRIBUTIONS OF SKIN BIOPSY TO DIAGNOSIS

Quantitation of epidermal nerve fiber density and morphology has been helpful for evaluating several clinical disorders.

Painful Sensory Neuropathy

In 20 patients with painful sensory neuropathies, intraepidermal nerve fiber density was significantly reduced compared with age-matched controls, even in regions proximal to the areas of clinically identifiable sensory abnormalities. For example, patients with grade 1 neuropathy in whom sensory abnormalities were restricted to the toes and feet had significantly reduced intraepidermal nerve fiber densities at the calf. Cooling thresholds on QST (CASE IV device) did not correlate with intraepidermal nerve fiber density or the clinical grade of sensory neuropathy (Fig. 34–15). Intraepidermal nerve fiber density from calf and thigh correlated against clinical estimates of neuropathy severity.[35]

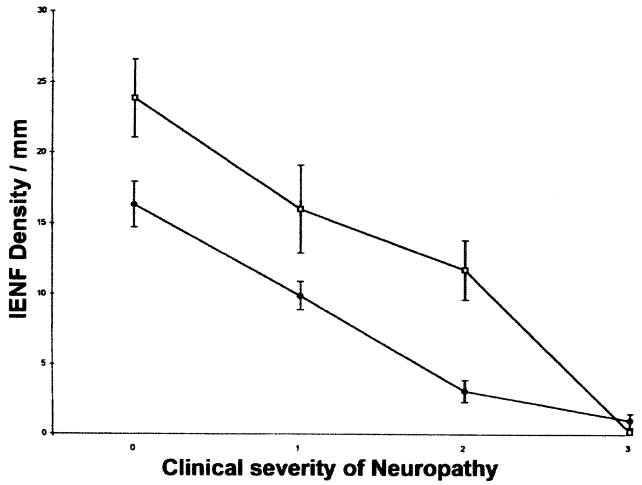

FIGURE 34–15
Intraepidermal nerve fiber density from calf (*black circles*) and thigh (*white squares*) skin correlated against clinical estimates of neuropathy severity in patients with painful sensory neuropathy. The data shown are group means with standard error bars. (From Holland, N. R., Stocks, A., Hauer, P., et al.: Intraepidermal nerve fiber density in patients with painful sensory neuropathy. Neurology *48*:708, 1997, with permission.)

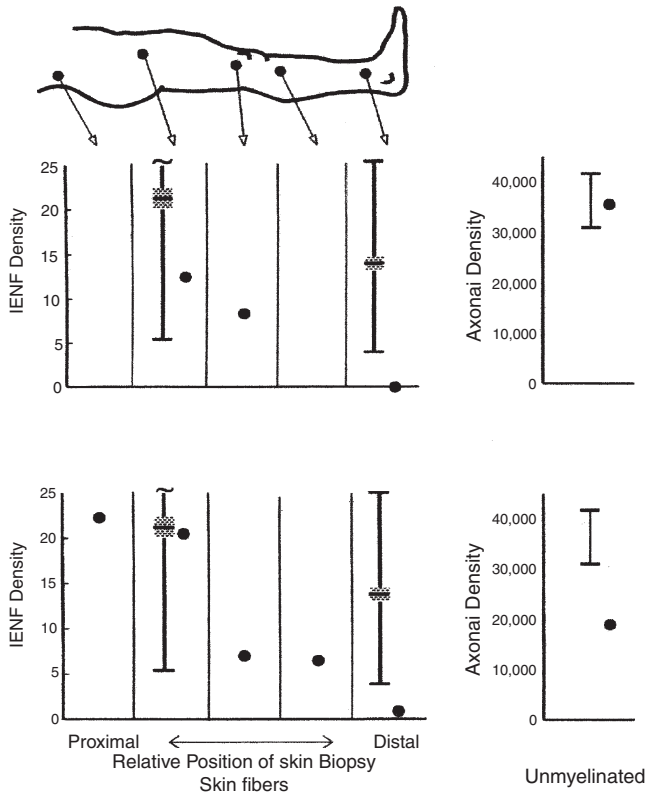

FIGURE 34–16
Intraepidermal nerve fiber (IENF) densities at various sites for chronic progressive idiopathic small fiber sensory neuropathy (SFSN) (**left**) and sural nerve morphometry for chronic progressive idiopathic SFSN patient 1 (**top right**) and patient 2 (**bottom right**). **Left**, IENF density (mm^{-1}) at various sites for patients compared with normal controls (mean ± standard error shown by *shaded rectangle* and 5th and 95th percentile range shown by *bars*). **Right**, Unmyelinated fiber density (axons/mm^2) for patients compared to the 5th and 95th percentile range for normal controls (*bars*). Patient 1 (**top**) had normal densities of myelinated and unmyelinated fibers in the sural nerve biopsy specimen (taken from the calf) despite marked cutaneous denervation in skin biopsies. Patient 2 (**bottom**), with more severe progressive idiopathic SFSN, had reduced densities of small-diameter myelinated and unmyelinated axons in the sural nerve biopsy specimen (taken from the ankle) in addition to marked length-dependent cutaneous denervation. (From Holland, N. R., Stocks, A., Hauer, P., et al.: Intraepidermal nerve fiber density in patients with painful sensory neuropathy. Neurology *48*:708, 1998, with permission.)

The clinical features, natural history, and neuropathology were described for 32 patients presenting with "burning feet," thought to represent idiopathic small fiber sensory neuropathies. Two clinical patterns were apparent based on natural history and anatomic location of cutaneous denervation. Most patients (28 of 32) presented with neuropathic pain that was initially restricted to the feet and toes, but eventually extended more proximally to involve the legs and hands. Intraepidermal nerve fiber density was most severely reduced distally, with more normal intraepidermal nerve fiber densities at proximal sites. The minority (4 of 32) presented with abrupt onset of generalized cutaneous burning pain and hyperesthesia. In these patients intraepidermal nerve fiber densities were reduced at both proximal and distal sites. For the entire group, intraepidermal nerve fiber densities were reduced at the calf site below the fifth percentile in 81% of all patients.[34] Figure 34–16 compares nerve densities in biopsies of the skin and sural nerve from one patient from each group.

Skin biopsy was obtained in a separate study of 57 patients with painful, burning feet; minimal signs of neuropathy; and normal nerve conduction studies. Of these, 44 (77%) had a reduced number of intraepidermal nerves at the distal calf. Some nerves ended abruptly just below the basement membrane; others had enlarged, swollen terminal nerve endings. Reduced ankle reflexes were found in 11%, pinprick loss on toes and feet in 45%, and loss of vibration sense in 43%. Only three patients had conditions known to be associated with peripheral neuropathy.[78]

Diabetic Neuropathy

Earlier work in painful diabetic neuropathy suggested that there was a predominance of involvement of unmyelinated and small myelinated nerve fibers in the sural nerve.[8] Small fibers have long been recognized to be involved in diabetic polyneuropathy, and skin biopsies have confirmed that epidermal denervation can occur relatively early, and generally

in a length-dependent manner. The total length of epidermal nerve per volume of epidermis correlates with the overall clinical severity (Fig. 34–17). Some of the surviving epidermal nerves have morphologic abnormalities, particularly axon swellings.[47,80] The subepidermal neural plexus is often thinner than in control skin, and fibers within the subepidermal plexus may have a thickened, dystrophic appearance. In some neuropathies single nerve fibers are seen ending in a terminal enlargement just under the basement membrane.[46] These fibers appear to be unsuccessfully attempting to penetrate basement membrane en route to regeneration into the epidermis, although it is also possible that they are in the process of dying back. Sweat gland innervation is sometimes abnormal or completely lost in diabetic patients (Fig. 34–17), but it appears not to be useful as an early indication of neuropathy.[46]

Epidermal nerve fibers also appear to be prominently affected in patients with impaired glucose tolerance (IGT). Several groups have reported an increased prevalence of IGT among patients with painful sensory neuropathy,[74,97] and randomly selected patients with sensory neuropathy and IGT were found to have reduced epidermal nerve fiber densities.[99] Of 97 patients with clinically diagnosed predominantly sensory neuropathy of undetermined etiology evaluated with oral glucose tolerance tests, 36% were classed as having IGT and 20% as having diabetes. Both the IGT and diabetes groups had reduced distal leg intraepidermal nerve fiber densities. The neuropathy associated with IGT was less severe than the neuropathy associated with diabetes. Patients with predominantly small fiber neuropathy had a shorter duration of neuropathy symptoms, and most had IGT. The results suggest that small-caliber nerve fiber loss may be the earliest detectable sign of neuropathy in glucose dysmetabolism.[103]

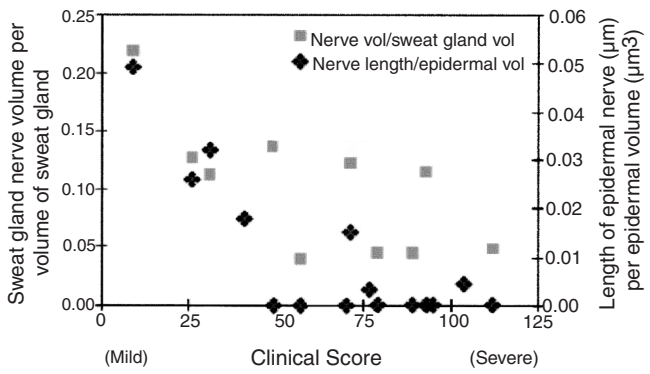

FIGURE 34–17

Neuropathy score correlated with epidermal nerve fiber density and sweat gland innervation density in diabetic subjects. Correlation of cutaneous nerve quantitation with clinical assessment. The amount of nerve in calf biopsies correlated with the clinical evaluation of most diabetic subjects. The data suggest that the epidermal nerve assessment is useful in evaluating mild to moderate neuropathy, and sweat gland nerve analysis is helpful in later stages of neuropathy. Reduction of epidermal nerve is seen in mild cases of neuropathy, whereas no epidermal nerve is seen in severe cases. Sweat gland innervation remains at normal levels until neuropathy is quite severe. The peripheral and autonomic systems of the diabetic subjects were evaluated from graded results of history and examination, nerve conduction, cardiorespiratory reflexes, warm and cold sensitivity, and sweat testing. (Kennedy, W. R., and Wendelschafer-Crabb, G.: Utility of skin biopsy in diabetic neuropathy. Semin. Neurol. *16*:163, 1996, with permission.)

Diabetic Truncal Neuropathy

In diabetic truncal neuropathy, skin biopsies from symptomatic regions show a loss of intraepidermal nerve fibers. After clinical recovery there may be nerve fiber regeneration.[53]

HIV-Associated Sensory Neuropathies

Sensory neuropathies of HIV/AIDS affect at least 30% of individuals with AIDS.[90] The distal sensory polyneuropathy associated with HIV overlaps clinically with the toxic neuropathy provoked by specific antiretrovirals. The sensory neuropathies produce paresthesias or burning pain in the feet, often associated with hyperalgesia and lightning pains. Unlike HIV-associated dementia, the incidence rates have not declined with the advent of highly active antiretroviral therapy, probably because specific dideoxynucleoside analogues (ddI, d4T, and ddC) produce peripheral neurotoxicity.[66] Punch skin biopsies were originally developed at the Johns Hopkins University laboratory to study HIV-associated sensory neuropathies that had previously been shown by sural nerve biopsy to be associated with prominent involvement of unmyelinated nerve fibers.[17] The most systematic survey of skin biopsies in HIV-associated sensory neuropathy comes from a trial of recombinant human NGF. Skin biopsies were included in this trial as an outcome measure, the first reported application of skin biopsy to measure treatment effect. Sixty-two of 270 patients with sensory neuropathies who participated in the trial of recombinant human NGF were included in a substudy examining the density of intraepidermal nerve fibers.[62] Fiber density was inversely correlated with neuropathic pain as measured both by patient and physician global pain assessments, but not using the Gracely Pain Scale. The reproducibility of the technique, as assessed from intrasubject correlation between baseline and the week 18 densities, was 81% in the distal part of the leg and 77% in the upper thigh. Decreased intraepidermal nerve fiber density at the distal leg was associated with lower CD4 counts and higher plasma HIV RNA levels. There was no treatment effect over the relatively short period of 18 weeks, and a few biopsies studied out to 70 weeks remained stable over time. Although no significant treatment effect was observed over the 18 weeks, the reproducibility of the technique appears to be good, suggesting that this technique could be incorporated as an outcome measure in future trials of regenerative agents for sensory neuropathies.

Friedreich's Ataxia

Friedreich's ataxia is an autosomal recessive inherited ataxia. Hyperexpression of a GAA trinucleotide on the causative gene (*X25*) localized to chromosome 9 results in reduction of the protein frataxin.[9] Pathology of large-diameter myelinated nerve fibers in peripheral nerves is consistent with the loss of vibratory sense and deep tendon reflexes and severely reduced sensory action potentials. Small fiber involvement was postulated but unproven[19] until severe loss of unmyelinated sensory nerves and of the autonomic innervation to sweat glands, arrector pili, and arterioles was uncovered in thick sections of skin biopsies.[70] The small fiber loss correlated with reduced mechanical and thermal nociception.[71]

Restless Legs Syndrome

The restless legs syndrome (RLS) is characterized by a compelling urge to move the extremities. It is often associated with paresthesias or dysesthesias, motor restlessness, worsening of symptoms with rest, relief by activity, and worsening of symptoms in the evening or night. Two forms are described: crurum dolorosum, characterized by pain, and crurum paresthetica, characterized by paresthesias.[21] Numerous risk factors are reported for RLS, and there is a suggestion that early-onset (age 45 or younger) and late-onset RLS differ etiologically.[1,2] Nerve biopsies performed in an unselected group of primary patients suggest that the incidence of peripheral neuropathy may be underestimated.[37] Twenty-two consecutive patients with RLS were evaluated for evidence of large fiber neuropathy (LFN) and small sensory fiber loss (SSFL). Neuropathy was identified in eight (36%). Three patients had pure LFN, two had mixed LFN and SSFL, and three had isolated SSFL. Patients with SSFL had a later onset, reported pain in their feet more frequently, and tended not to have a family history of RLS. Patients without SSFL did not associate onset with age, family history, or presence of pain. The report suggested that two forms of RLS exist: one is triggered by painful dysesthesias associated with SSFL and has a later onset and a negative family history; the other does not have small sensory fiber involvement, has an earlier onset and a positive family history, and is without pain.[79]

Familial Dysautonomia (Riley-Day Syndrome, Hereditary Sensory and Autonomic Neuropathy Type III)

Familial dysautonomia[3,86] is an autosomal dominant disorder of sensory and autonomic nerves found in Ashkenazi Jews that is caused by mutation of the *IKBKAP* gene on chromosome 9q31.[6] Clinical features include increased sweating, absent overflow lacrimation, vomiting crises, decreased sensitivity to pain, depressed Achilles reflexes, postural hypotension, and absence of tongue fungiform papillae and of the axon flare response to intradermal histamine. Biopsy of glabrous skin shows severe loss of unmyelinated sensory and autonomic nerves at the calf and moderate loss in the paraspinal region of the upper back.[33]

Congenital Insensitivity to Pain with Anhidrosis (Hereditary Sensory and Autonomic Neuropathy Type IV)

Congenital insensitivity to pain with anhidrosis (CIPA) is a rare condition caused by mutation of the TrkA (*NTRK1*) gene on the 1q 21-22 chromosome,[38] characterized by mental retardation; congenital analgesia that leads to self-mutilation, multiple scars, and fractures; and anhidrosis with repeated bouts of fever.[87] Nerve biopsy shows loss of unmyelinated and small myelinated fibers.[25] Recently a 10-year-old girl was reported with a low IQ, pain, and thermal insensitivity. The remainder of the neurologic examination, sensory nerve conduction velocities, cardiovascular reflexes, and visual, brainstem, and somatosensory evoked potentials were normal. Sural nerve biopsy was devoid of small myelinated nerves and contained only rare unmyelinated nerves. Epidermal nerve fibers were essentially absent in skin from the thigh, back, and calf. The few sweat glands present were hypotrophic and without innervation.[69]

Psoriasis

Psoriasis is a common skin disorder characterized histologically by epidermal hyperproliferation, dilated tortuous capillaries, and a lymphocytic infiltrate. Skin biopsy shows that the epidermal nerves are greatly elongated through a thickened epidermis to the stratum corneum.[39] Several treatment modalities are available: therapy that targets epidermal proliferation, immunomodulators, or physical treatment, such as the use of lasers. The pulsed dye laser at 585-nm wavelength targets blood vessels and selectively destroys dilated vessels in the dermal papillae. Laser therapy is followed by remodeling of the epidermis and subsequent regeneration of capillaries and epidermal nerves with return to a more normal appearance.[118]

Port-Wine Stains

It is proposed that the pathogenesis of port-wine stains might be related to a lack of innervation around the ectatic blood vessels.[89] Biopsy of the involved skin shows a deficiency of innervation that is inversely proportional to the size of the vascular spaces. Moreover, nerve density may be predictive of the response of port-wine stain lesions to laser treatment.[117]

Leprosy

Severe loss of immunoreactivity to PGP 9.5 and neurofilament antibodies occurs in leprosy. Loss is greatest in the tuberculoid variety of leprosy, less in lepromatous, and least in the indeterminate variety.[41] Curiously, there is almost complete loss of reactivity for the neuropeptides CGRP, SP, and neuropeptide Y. This raises the possibility that neuropathy may be detected early by loss of immunoreactivity to some antigens while reactivity to other antigens remains, at least temporarily.

Fabry's Disease

Fabry's disease is an X-linked recessive disorder caused by deficiency of α-galactosidase A activity. Hemizygotes develop deposits of neutral glycosphingolipids, principally ceramide trihexoside, throughout the nervous system but predominantly in vascular endothelial cells. A painful small fiber neuropathy develops that is difficult to detect and quantitate by conventional methods. The neuropathy can develop in children as young as 5 years of age, with characteristic episodes of acral burning pain. Twenty Fabry's disease patients (hemizygotes, ages 19 to 56 years) with preserved renal function were found to have normal nerve conduction studies and large fiber quantitation by sural nerve biopsy. By contrast, involvement of small cutaneous fibers in these patients was easily demonstrated and quantified by punch skin biopsy. All patients showed severe loss of intraepidermal innervation at the distal part of the leg, with a density of 0 to 2.4 fibers/mm versus a density for control subjects at this site of 4.7 to 6.5 fibers/mm. Fiber loss at the distal thigh was proportionately less severe. Fabry's disease patients underwent biopsy at 6-month intervals for periods ranging from 1 to 3 years with no significant longitudinal changes in density except in two patients who demonstrated a rapid decrement in innervation density following an increase in spontaneous pain.[92]

Sensory Ganglionopathies

Ganglionopathy patients tend not to show the normal gradient in intraepidermal nerve fiber density between the proximal thigh and the distal part of the leg—that is, they show a non–length-dependent process. One cohort presented with predominant gait and limb ataxia and proprioceptive sensory loss; 8 of 16 patients had positive sensory symptoms. Causes of the ganglionopathy were paraneoplastic in six and idiopathic in seven, and one had a hereditary sensory and autonomic neuropathy. In ganglionopathies, the mean intraepidermal nerve fiber density did not differ between the proximal thigh (10.37/mm) and the distal leg (10.41/mm). Compared to other sensory neuropathies and controls, the intraepidermal nerve fiber density was significantly lower at the proximal thigh site in patients with ganglionopathies.[54]

Postherpetic Neuralgia

Shingles represents reactivation of latent varicella-zoster virus in a sensory ganglion, causing loss of a variable proportion of the sensory neurons. In some patients, neuropathic pain persists for months or years (postherpetic neuralgia). Hyperalgesia and allodynia to light touch are often prominent. Two studies have demonstrated that the density of epidermal nerves in areas of allodynia is reduced, although they came to differing conclusions. One study found that higher fiber densities correlated with pain, an observation that might suggest that intact but hyperactive nociceptors were responsible. The reduction in intraepidermal nerve fiber density correlated with the magnitude of thermal sensory deficits.[88] By contrast, a second study[75] found that pain correlated with lower intraepidermal nerve fiber densities, and interpreted the results as more compatible with central sensitization. Interestingly, there was also a reduction of intraepidermal nerve fiber density in the contralateral, unaffected epidermis.[115]

CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL) is one example of how skin biopsy can be used for analysis of nonneural substances in investigation of neurologic disease. CADASIL is an autosomal dominant disorder that presents as migrainous headaches, cerebral ischemia, and multi-infarct dementia. An abnormality on chromosome 19q12 produces a missense point mutation in the *notch3* gene. Punch skin biopsy has been used to identify characteristic granular, electron-dense, osmiophilic material attached to vascular smooth muscle cells.[109] However, a commercial laboratory test for the genetic abnormality is now available from Athena Labs (Worcester, MA), which should provide improved specificity and sensitivity.[30]

Chronic Inflammatory Demyelinating Polyneuropathy

Patients with chronic inflammatory demyelinating polyneuropathy (CIDP) were recently shown to have about 50% reduction in intraepidermal nerve fiber density compared to age- and gender-matched controls, suggesting that small-diameter sensory nerves as well as large-caliber nerve fibers are affected in CIDP.[15]

Postural Tachycardia Syndrome

A collaborative study by Johns Hopkins University and the Mayo Clinic examined the relationship between the QSART and epidermal nerve fiber densities in patients with postural tachycardia syndrome (POTS).[96] Inclusion criteria for POTS in the study included orthostatic heart

rate increment (from 30 beats/min to an absolute heart rate of 120 beats/min within 5 minutes of head-up tilt), symptoms of orthostatic intolerance, distal abnormal QSART, and abnormalities or loss of vasoconstrictor response. In POTS, distal autonomic fibers are affected in isolation, sparing somatic fibers, which have consistently normal nerve conduction studies.[91] Intraepidermal nerve fiber density correlated with peripheral postganglionic sudomotor status as assessed by the Composite Autonomic Severity Score (CASS)[102] both in the category of the sudomotor subscores and in the overall total score, which is a composite index of combined sudomotor, adrenergic, and cardiovagal function. In POTS the mean intraepidermal nerve fiber density was normal, while the CASS was abnormal. Although three of the eight patients showed some minor morphologic abnormalities, the normalcy of the epidermal nerve fiber densities suggests that distal involvement of autonomic fibers occurs in isolation in POTS.

Pediatric Neurologic Disorders

Skin biopsies have been less widely studied in pediatric neurologic disorders, but there are some conditions in which examination of the cutaneous innervation has characteristic changes. Two examples are provided.

Giant Axonal Neuropathy

Giant axonal neuropathy is an autosomal recessive neurologic disorder characterized by the development of a severe polyneuropathy, central nervous system abnormalities, and characteristic tightly curled hair. Mutations in the gigaxonin gene have been identified as the underlying genetic defect.[51] Ultrastructurally, accumulations of neurofilaments and osmiophilic aggregates are found in giant axons. Intermediate filaments accumulate in Schwann cells, perineural cells, fibroblasts, and endothelial and epithelial cells in both nerve and skin biopsies.[104] We have observed giant swellings within dermal nerve fibers, although the density of epidermal nerves appears to be normal.

Neuroaxonal Dystrophies

Neuronal intranuclear inclusion disease has no defined metabolic abnormalities, but it affects the peripheral nervous system, which could therefore be used to identify morphologic abnormalities.[24] In infantile neuroaxonal dystrophy, there is also an accumulation of intermediate neurofilaments, as well as mitochondria and organelles with vesiculotubular profiles.[59] There is limited experience with the use of skin biopsies in this condition; however, some children have shown dystrophic changes in the subepidermal neural plexus.

Other Conditions

Depletion of epidermal nerve fibers has been demonstrated in a variety of conditions with dermatologic manifestations. Image analysis detected a significant decrease in PGP 9.5–immunoreactive nerves in the epidermis and subepidermal layers and in CGRP-immunoreactive nerves around the capillaries within dermal papillae of patients with Raynaud's phenomenon, although the changes were not always apparent by visual screening.[105] Patients with systemic sclerosis were found to have decreased CGRP- and PGP 9.5–immunoreactive nerves in all skin areas and vasoactive intestinal polypeptide–immunoreactive nerves in the sweat glands.[105] An increased number of PGP 9.5–immunoreactive fibers has been reported in the dermis and dermal-epidermal junction in atopic dermatitis, a condition in which the sensation of itching is prominent.[108] It has been suggested that increased dermal innervation could explain the pruritus, burning pain, and hyperalgesia in notalgia paresthetica,[100] and that an increase of sensory neuropeptides SP and CGRP might be involved in the pathogenesis of nodular prurigo.[64] No changes of innervation were found in patients with lichenified eczema.[64]

RESEARCH USES OF SKIN BIOPSY

The existence of unmyelinated sensory nerves within a few microns of the skin's surface has provided opportunities to study the reactions of peripheral nerves to mechanical, thermal, and chemical stimuli under safe, minimally invasive circumstances. Early experiments, using methylene blue staining, localized the itching sensation caused by cowphage spicules and injected proteases to the subepidermal neural plexus.[93] Use of immunohistochemical staining has allowed expansion of research into the correlates of sensory testing, effects of mechanical and chemical trauma to cutaneous nerves, and regeneration of sensory nerves.

Human Models of Nerve Regeneration

Skin Biopsy Variations

We developed two models to study reinnervation of the epidermis.[83] One model uses a circular incision that transects the subepidermal plexus, resulting in Wallerian degeneration of the nerve fibers that enter the incised cylinder, leaving a defined zone of denervated dermis and epidermis. The earliest reinnervation of epidermis occurred by collateral sprouting from the terminals of epidermal axons from just outside the incision line. These terminals extended horizontally across the incision line and through the superficial layers of the epidermis, beneath the stratum corneum. By 13 days, numerous regenerating axons appeared in the deeper dermis derived

from transected axons. The latter regenerating axons grew toward and ultimately into the epidermis, so that epidermal axonal density had normalized by 30 to 75 days. The invasion of these axons was associated with regression of the horizontally growing collateral sprouts. The second model utilizes an identical incision followed by removal of the incised cylinder of skin, leaving a denervated area in which Schwann cells are absent. New fibers arose by terminal elongation of the epidermal axons outside the incision line, as in the incision model, and especially by collateral branching of epidermal fibers at the incision margins. These collaterals reached the epidermal surface of the basal lamina at the dermal-epidermal junction, and then grew slowly toward the center of the denervated circle. In contrast to the first model, complete reinnervation was not achieved even after 23 months. These and other models might be used to study reinnervation of denervated skin in humans in different injury models and have relevance for exploring the stimuli for axonal growth and remodeling.

Skin Blister: A Model of Mechanical Denervation

Mechanical denervation of the terminal 40 to 60 μm of epidermal sensory nerve fibers can be produced to study wound healing and sensory reinnervation by removing the epidermis with a skin blister.[49] Regeneration of new epidermis occurs within 3 days after a 3-mm blister wound. The new epidermis is initially innervated by collateral sprouts arising from epidermal nerve fibers in the normal skin surrounding the blistered area. Slightly later, the proximal stumps of epidermal nerves at the base of the blister begin to enter the new epidermis, first near the edge of the lesion and later at the center. Eventually, regenerated nerves from the proximal stumps in the base reinnervate the epidermis in near-normal numbers and the collateral branches disappear.[112] Reinnervation is accompanied by return of sensation within 30 days. Similar reinnervation occurs in patients with diabetic neuropathy, even in areas of reduced sensation, but the return of epidermal nerve fibers is only to the level of the prelesion innervation.[42]

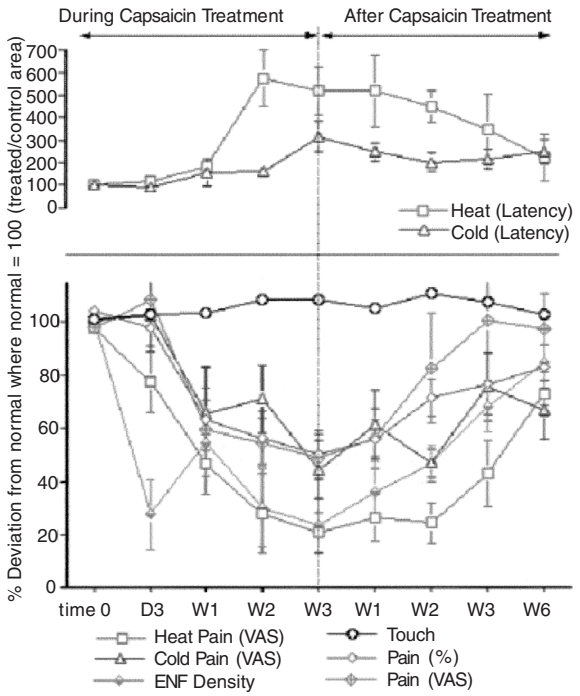

FIGURE 34–18

Capsaicin-induced denervation and reinnervation. The mean (± standard error) change in sensation and number of nerve fibers expressed as percent deviation from normal during and following the course of topical capsaicin application. Note that scale for latencies (**top**) is different from that for other sensitivities and nerve fiber numbers (**bottom**). Touch was measured using Semmes Weinstein monofilaments. Intensity of pain was measured using a visual analogue scale (V.A.S.) for mechanical pain, heat pain, and cold pain. Mechanical pain (pain % and pain V.A.S.) was evaluated with a spring-mounted pin. Heat pain (V.A.S.) and cold pain (V.A.S) were tested with a 2-mm probe. Epidermal nerve fiber (ENF) number per area was calculated from analysis of nerves in blister roofs. (From Nolano, M., Simone, D. A., Wendelschafer-Crabb, G., et al.: Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. Pain *81*:135, 1999, with permission.)

Capsaicin: A Model for Chemical Denervation

When capsaicin, a pungent ingredient in hot chili peppers, is applied topically, it causes warm, painful sensations. These are replaced after several hours by a period of hyposensitivity. Sensation returns toward normal in 2 to 3 weeks.[95] This sequence was initially believed to result from desensitization of nociceptive receptors but was later shown to be induced by the influx of calcium ions with osmotic changes as well as activation of calcium-sensitive proteases resulting in axonal degeneration.[113] More recently, skin biopsy showed that capsaicin administered intradermally is soon followed by depletion of epidermal nerves and superficial dermal nerves within 24 hours (Fig. 34–18). Reinnervation of the epidermis reached approximately 50% of normal, and heat pain sensation reached 60% of normal after 4 weeks.[94] Capsaicin administered topically also causes loss of epidermal and superficial dermal nerves and decreased sensitivity to noxious heat and mechanical (pin) stimulation. Serial biopsies reveal that nerve regeneration after topical capsaicin is faster than after intradermal delivery. The return of epidermal nerves and sensation is nearly complete in 30 days (Fig. 34–19).[72] These findings strengthen the hypothesis that epidermal nerves are polymodal nociceptors. The detection of a loss and subsequent recovery of sensitivity to noxious heat was possible when stimuli were delivered by a small probe (2- to 3-mm diameter) but not by the larger 30 × 30-mm probe of the type common on

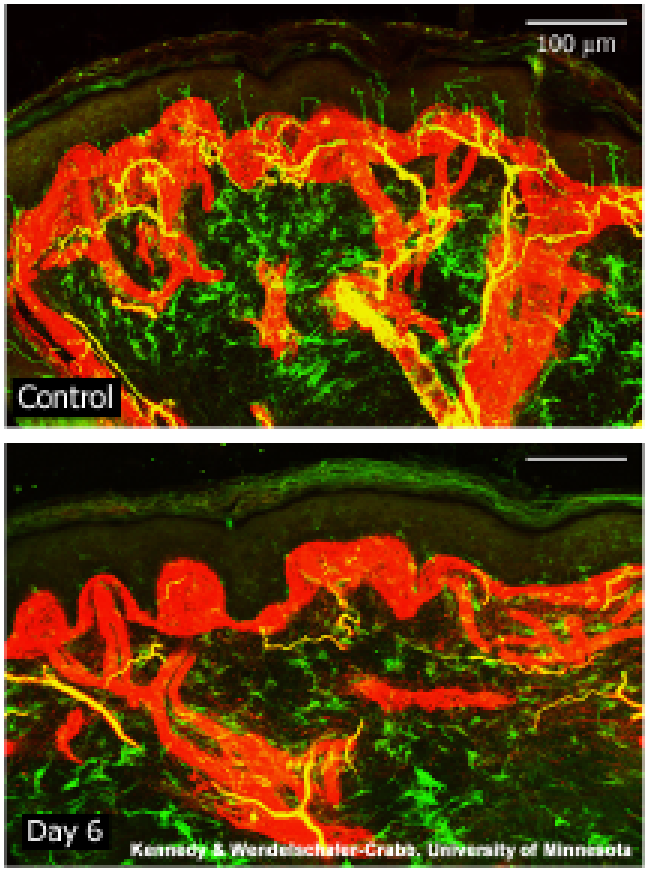

FIGURE 34–19
Capsaicin-treated human epidermis. Nerves degenerate following topical capsaicin treatment, as can be seen by comparing control tissue at time 0 (**top**) with a sample taken after 1 week of capsaicin application (**bottom**). Epidermal nerve fibers have degenerated, while fibers in the subepidermal neural plexus are still plentiful. This provides a good model for the peripheral neuropathy seen in diabetic subjects, in whom epidermal nerve fiber loss precedes more proximal loss. We will use this model to study basement membrane during nerve regeneration. This model provides denervation without remodeling basement membrane. Bar: 100 µm. *See Color Plate*

commercial instruments.[50] Both of our groups have developed reproducible systems for applying capsaicin and examining the patterns and rate of regrowth of epidermal nerve fibers. In normal volunteers treated with topical capsaicin, epidermal nerve density returns at an average rate of 0.177 fibers/day (range, 0.023 to 0.362 fibers/day). The rate of nerve fiber regeneration is significantly decreased in diabetics (0.074 fibers/day; range, 0.002 to 0.258 fibers/day, $P < .001$), and there was a trend for a lower rate of regeneration with increasing severity of neuropathy (Fig. 34–20).[81a] These models have potential as outcome measures in trials of regenerative agents and for improving understanding of trophic factor regulation after nerve injury.

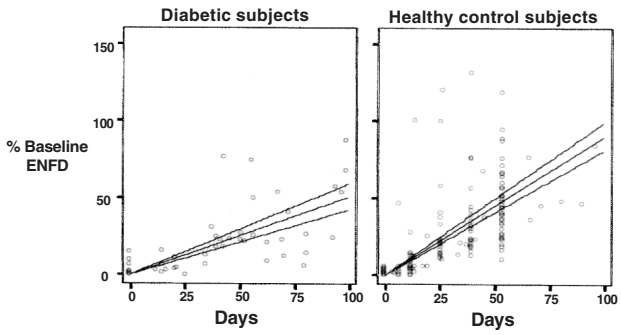

FIGURE 34–20
ENFD regeneration expressed as a percent of baseline values in diabetic and healthy, nondiabetic control subjects. Healthy volunteers recovered their ENF density at a rate of 0.177 ± 0.075 fibers/mm/day. The rate of nerve fiber regeneration was significantly decreased among those with diabetes (0.074 ± 64 fibers/mm/day; $P < .001$) and further reduced among diabetic subjects with neuropathy as compared to those without neuropathy ($P = .003$). (From Polydefkis, M., Hauer, P., Sheth, S., et al.: The time course of epidermal nerve fiber regeneration: Studies in normal controls and in people with diabetes, with and without neuropathy, Brain *127*:1606, 2004, with permission.)

Comparison of Nerve Function with Structure

Comparisons have been made between the morphology of cutaneous nerve receptors and the results of electrophysiologic stimulation. Highly significant correlations were found between the density of normal Meissner's corpuscles and proximally recorded sensory nerve action potential areas whether generated by electrical stimulation of the digital nerve or by tactile stimulation of the fingerpad. The density of abnormal Meissner's corpuscles correlated significantly with the sensory action potential area but not with tactile potential area, whereas the density of papillary myelinated nerve fibers correlated with tactile but not with electrical stimuli. The natural stimuli appeared to be transmitted centrally more effectively by normal receptors than by atrophic receptors.[71]

Animal Models Using Skin Biopsy

Several groups have now used skin biopsy techniques in animal models to examine denervation and regeneration after various surgical models of nerve injury.[67,68,36,56,115] Early experiments showed that rat sciatic nerve transection resulted in denervation, thinning of the epidermis, and increased expression of PGP 9.5 by Langerhans cells.[36]

A mouse model has been devised to correlate the reinnervation time of muscles, sweat glands, and skin with the time required for return of function to these organs (Fig. 34–21). After sciatic nerve crush, sequential biopsies of foot muscles and footpads from the hind paw of the same mouse can be examined to detect the time of reappearance of alpha motor, sudomotor, and epidermal nociceptor nerves. Reappearance

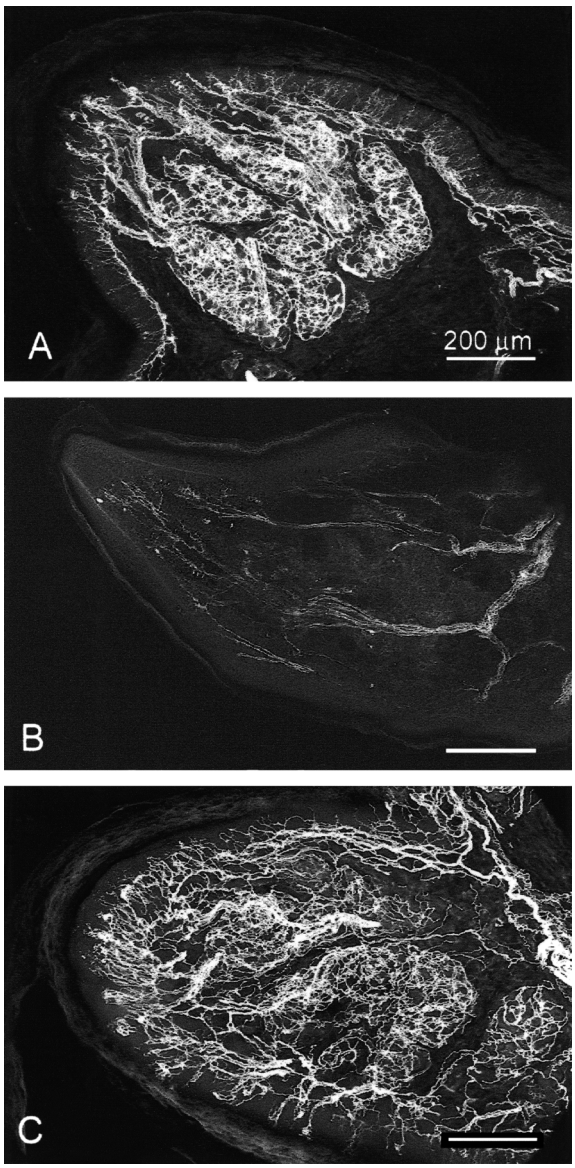

FIGURE 34–21
Confocal micrographs of PGP 9.5 immunofluorescence in control (**A**) and reinnervating (**B** and **C**) mouse footpads following sciatic nerve crush. **A,** Nerves in control footpad form a dense network. Sweat glands are very heavily innervated. Large trunks extend through the central sweat gland area to form the subepidermal nerve plexus and Meissner's corpuscles in the tufts of papillary dermis. The epidermis has many fine nerve endings. **B,** At day 14 postcrush, PGP 9.5–immunoreactive fibers returned in large nerve trunks from the base of the pad to the papillary dermis. **C,** By day 46 postcrush, the pattern of innervation resembles the control, but regenerated nerve fibers to the nerve trunks, subepidermal neural plexus, and sweat glands are less well compartmentalized, and the epidermis has shorter nerve fibers. (From Navarro, X., Verdu, E., Wendelschafer-Crabb, G., and Kennedy, W. R.: Immunohistochemical study of skin reinnervation by regenerative axons. J. Comp. Neurol. *380*:164, 1997, with permission.)

of end-organ function can be tested at the same time intervals by sciatic nerve stimulation recording muscle, pilocarpine (intraperitoneal) stimulation to detect reappearance of sweat droplets, and pinprick to skin to detect return of nociception.[68] The model has potential for testing the efficacy of therapeutic agents to facilitate nerve regeneration.

Reinnervation of epidermal nerves in pigskin was tested after removal of a 3-mm skin biopsy and immediate replacement (autotransplantation). The first nerves that entered the newly regenerated epidermis over the wound were collateral branches of epidermal nerves in the normal uninjured epidermis surrounding the wound. These nerves grew on the epidermal surface of the basement membrane toward the center of the wound, then turned at an acute angle toward the stratum corneum. Nerve regeneration from the severed subepidermal plexus nerve trunks along the sides of the lesion began much later (weeks) and extended slowly toward the center of the lesion. Reinnervation never approached normal.[29] The same experiment on one human subject gave identical results (M. Nolano, personal observation, 1997).

An important experimental study demonstrated that intraepidermal nerve fiber density is significantly reduced after dorsal root ganglionectomy or sciatic nerve transection in rats, but not after dorsal and ventral radiculotomy, sympathectomy, or spinal motor neuron lesion.[56]

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