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This paper completely kills the argument that the receptive structures of our body are located "too deep" to be affected by EMFs from e.g. base stations or hand held gadgets, such as mobile phones.

Cell and Tissue Research

Protein gene product 9.5-immunoreactive nerve fibres and cells in human skin

Lixin Wang[1], Marita Hilliges[1], Tomas Jernberg[1], Desirée Wiegleb-Edström[2], and Olle Johansson[1]

[1] Department of Histology and Neurobiology, Karolinska Institutet, and
[2] Department of Dermatology, Karolinska Hospital, Stockholm, Sweden

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Summary. Sections of human skin were processed according to the indirect immunofluorescence technique with a rabbit antiserum against human protein gene product 9.5 (PGP 9.5). Immunoreactivity was detected in intraepidermal and dermal nerve fibres and cells. The intraepidermal nerves were varicose or smooth with different diameters, running as single processes or branched, straight or bent, projecting in various directions and terminating in the stratum basale, spinosum or granulosum. The density of the intraepidermal nerves varied between the different skin areas investigated. PGP 9.5-containing axons of the lower dermis were found in large bundles. They separated into smaller axon bundles within the upper dermis, entering this portion of the skin perpendicular to the surface. Then they branched into fibres mainly arranged parallel to the epidermal-dermal junctional zone. However, the fibres en route to the epidermis traversed the upper dermis more or less perpendicularly. Furthermore, immunoreactive dermal nerve fibres were found in the Meissner corpuscles, the arrector pili muscles, hair follicles, around the eccrine and apocrine sweat glands and around certain blood vessels. Such fibres were also observed around most subcutaneous blood vessels, sometimes heavily innervating these structures. Numerous weakly-to-strongly PGP 9.5-immunoreactive cells were found both in the epidermis and in the dermis.

Key words: PGP 9.5 (human protein gene product 9.5) – Skin – Immunohistochemistry – Nerve fibres – Dendritic cells – Merkel cells – Human

Recent developments in methods of separating protein mixtures have raised the possibility of detecting and quantifying all the estimated 30000–50000 protein gene products (PGPs) coded for by the human genome (Anderson and Anderson 1979). PGP 9.5 is a neurone-specific protein originally discovered by high-resolution two-dimensional mapping of soluble proteins from different human organs (Jackson and Thompson 1981; Doran et al. 1983). The primary protein structure opened the possibility for more refined immunohistochemical studies, and thus, the use PGP 9.5 as a general cytoplasmic marker of neurones and neuroendocrine cells, a diverse group of cells long noted to possess developmental similarities (Pearse 1969; Pearse and Polak 1972), which now also may be investigated at the molecular level (cf. Day and Thompson 1987). Immunohistochemistry has shown that this substance is widely distributed in central and peripheral nerve cells (Thompson et al. 1983), in the retina (Osborne and Neuhoff 1985), and in cells of the so-called diffuse neuroendocrine system (DNES). The distributional pattern has been claimed to be partly parallel to that of neurone-specific enolase (NSE), which is one of the few other general cytoplasmic markers known for such cell types (Schmechel et al. 1978).

Some PGPs are known to be primarily expressed in particular tissues and among these several proteins are known to be specific to the nervous system (Moore and Perez 1968; Bock 1978). Eight PGPs occurred almost exclusively in brain tissue (Jackson and Thompson 1981); these were PGPs 5.0, 5.5, 5.9a, 6.1, 8.4, 9.4, 9.5, and 11.7. By co-electrophoresis of the relevant purified brain proteins, PGP 5.5 was identified as $\gamma\gamma$-enolase (14-3-2 protein; NSE), PGP 5.9a as creatine kinase-BB, PGP 6.1 as aldolase C_4, and PGP 8.4 as 14-3-3 protein. The non-brain-specific proteins actin (PGP 6.0) and calmodulin (PGP 11.4) were also identified by co-electrophoresis. PGPs 5.0, 9.4, 9.5 and 11.7 appear predominantly in brain tissue. However, there was a faint trace of PGP 5.0 in large intestine, prostate and testis; of PGP 9.4 in adrenal, liver and testis; and of PGP 9.5 in kidney, large intestine, prostate and testis. PGP 11.7 occurred exclusively in brain.

Send offprint requests to: Olle Johansson, Department of Histology and Neurobiology, Karolinska Institutet, P.O. Box 60400, S-10401 Stockholm, Sweden

PGP 9.5 has also been used widely, together with such markers as protein S-100 and NSE, to study various types of tumours. For instance, Rode and collaborators (1985) studied human neuroendocrine tumours using PGP 9.5 as an immunohistochemically detectable marker substance. They felt PGP 9.5 was a valuable additional probe in the exploration of the paracrine system and for diagnosis of tumours arising from it. Springall and his collaborators (1986), in their elegant work on extra-pulmonary small cell carcinoma demonstrated, by use of immunohistochemistry, especially the use of PGP 9.5, NSE as well as the C-flanking peptide of human pro-bombesin as markers for tumour-derived cells. They were able to suggest, based on their results, that non-pulmonary small cell carcinoma has an endocrine character (Springall et al. 1986). Also ocular melanomas have been investigated using exactly these marker substances (Williams et al. 1987). Finally, testicular tumours and normal testicular tissue have been studied using PGP 9.5 immunohistochemistry (Hamid et al. 1986).

There is still no clear picture regarding the presence of neuroactive substances within nerve fibres in the human epidermis, apart from single reports of nerve terminals containing substance P, calcitonin gene-related peptide, somatostatin, galanin, etc., especially in the basal layer of the epidermal skin (for review, see Johansson 1987). Silver-impregnated nerves have been traced as far as to superficial layers of the human epidermis (Novotny and Gommert-Novotny 1988). In the present study, as a novel and at the same time general neurone-specific marker, PGP 9.5 was applied for a more complete demonstration of intraepidermal free nerve endings. Attention was also paid to the distribution of immunoreactive cellular elements in the dermis and at the epidermal/dermal junction. In parallel, we are also interested in using PGP 9.5 for quantitative studies concerning pathological changes in certain dermatologic disorders, such as pruritus. During the preparation of this paper, a similar study was recently published by Dalsgaard et al. (1989b).

Materials and methods

Skin punch biopsies (3–6 mm) were obtained from healthy volunteers, including samples from finger-tip, palm, upper arm, lower leg, chest and back. Lidocaine (0.5%) without epinephrine was used for local anaesthesia. After fixation in 4% paraformaldehyde and 14% saturated picric acid for 2 h at 4° C, the specimens were rinsed in phosphate-buffered saline (PBS) with 10% sucrose added for at least 24 h. Cryostat sections of 14 μm thickness were cut and thawed onto gelatine-coated slides.

The indirect immunofluorescence technique (see Coons 1958) was used for demonstrating PGP 9.5-immunoreactive elements. The sections were kept in a humid atmosphere, incubated with rabbit anti-human PGP 9.5 serum (1:1000, 1:2000 or 1:2500; Ultraclone, RA 95101) overnight at 4° C, rinsed in PBS, incubated for 30 min at 37° C in rhodamine (TRITC)- or fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:80 or 1:40; Boehringer Mannheim, FRG), rinsed and mounted. All the antisera were diluted in 0.3% Triton X-100. For observation and photography a Nikon Microphot FXA or Optiphot fluorescence microscope was utilized. For further technical aspects, see, e.g., Johansson and Nordlind (1984) and Johansson (1985).

Results

PGP 9.5 immunoreactivity was identified in nerve fibres and cells of the epidermis and the dermis. No obvious sex differences were encountered.

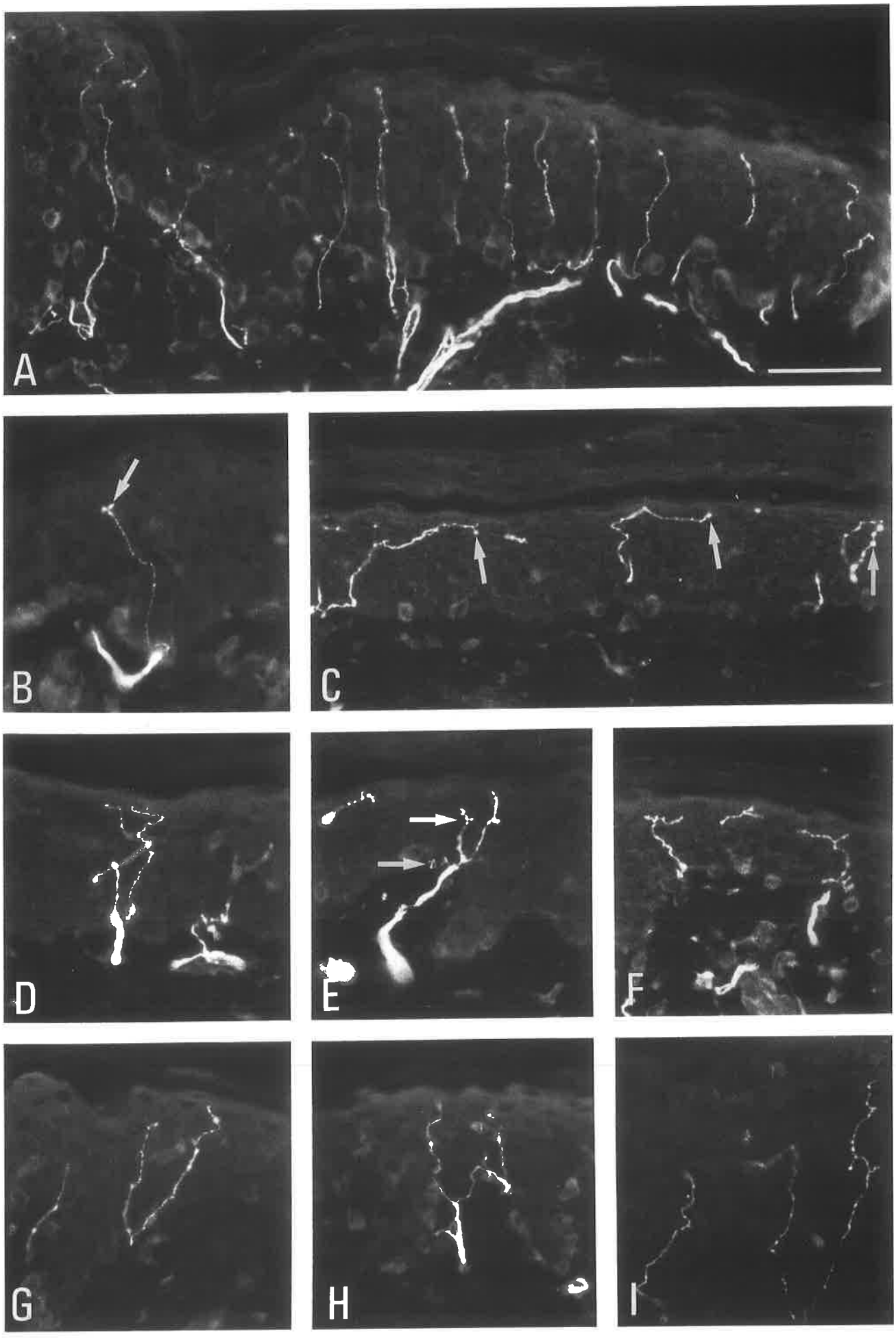
Nerve fibres

The intraepidermal nerve fibres were distributed throughout the stratum basale, spinosum and granulosum. They appeared as free endings, varicose or non-varicose, and entered the epidermis originating from small nerve bundles in the upper dermis. Then they took separate routes in the epidermis, some straight up to the superficial layers, some parallel to the skin surface within the different layers, and some running in a tortuous fashion. The PGP 9.5-immunoreactive nerves in the epidermis sometimes branched, stretching in different directions, and some ending in 'claw'-like or cone-shaped structures (Fig. 1). Distinct varicosities, partly large, 'knob'-like enlargements, occurred in the course of certain nerve fibres (Fig. 1B, C). The density of intraepidermal nerves was variable, however, the back appeared to possess more fibres, the extremities less. Furthermore, the intraepidermal nerves in the finger-tip and palm were less branched than those in other parts of the body.

In the dermis PGP 9.5-positive, strongly fluorescent, nerve bundles were observed (Fig. 2). These bundles ran perpendicular to the surface with the vessels to the superficial portion beneath the epidermis, extending parallel to the epidermal-dermal junctional zone or interweaving into nerve networks. However, the fibres en route to the epidermis traversed the upper dermis in a more or less straight, perpendicular fashion. The dermal papillae contained many PGP 9.5-positive nerves, especially in the palm and finger-tip, which gave off branches, mostly from the top of the papillae, into the epidermis. The nerve terminals in Meissner's corpuscles also showed PGP 9.5 immunoreactivity (Fig. 2F).

Arterioles in the lower dermis, in the upper and lower palmar dermis, and in the subcutis were innervated by dense neuronal networks. However, generally few vascular structures were innervated by PGP 9.5-immunoreactive nerves in the upper dermis (Fig. 3A, B). Dense nervous networks were detected around the external root sheath of the neck and lower part of the hair follicles. A special neural morphology was displayed between the muscle cells of the arrector pili. These nerves ran parallel to the smooth muscle cells and showed a wavy pattern (Fig. 3C). Abundant PGP 9.5-positive nerves were distributed around acini of eccrine sweat glands, however, not along the sweat ducts (Fig. 3D). Much less nerves were found around acini of apocrine sweat glands, and few, if any, around sebaceous glands.

Fig. 1A–I. PGP 9.5-immunoreactive nerves in human epidermis (indirect immunofluorescence technique). **A** Back; **B–E** lower leg; **F** chest; **G, H** upper arm; **I** fingertip. *Arrows* in **B** and **C** show 'knob'-like figures. *Arrows* in **E** show 'claw-like' endings. *Bar*: 50 μm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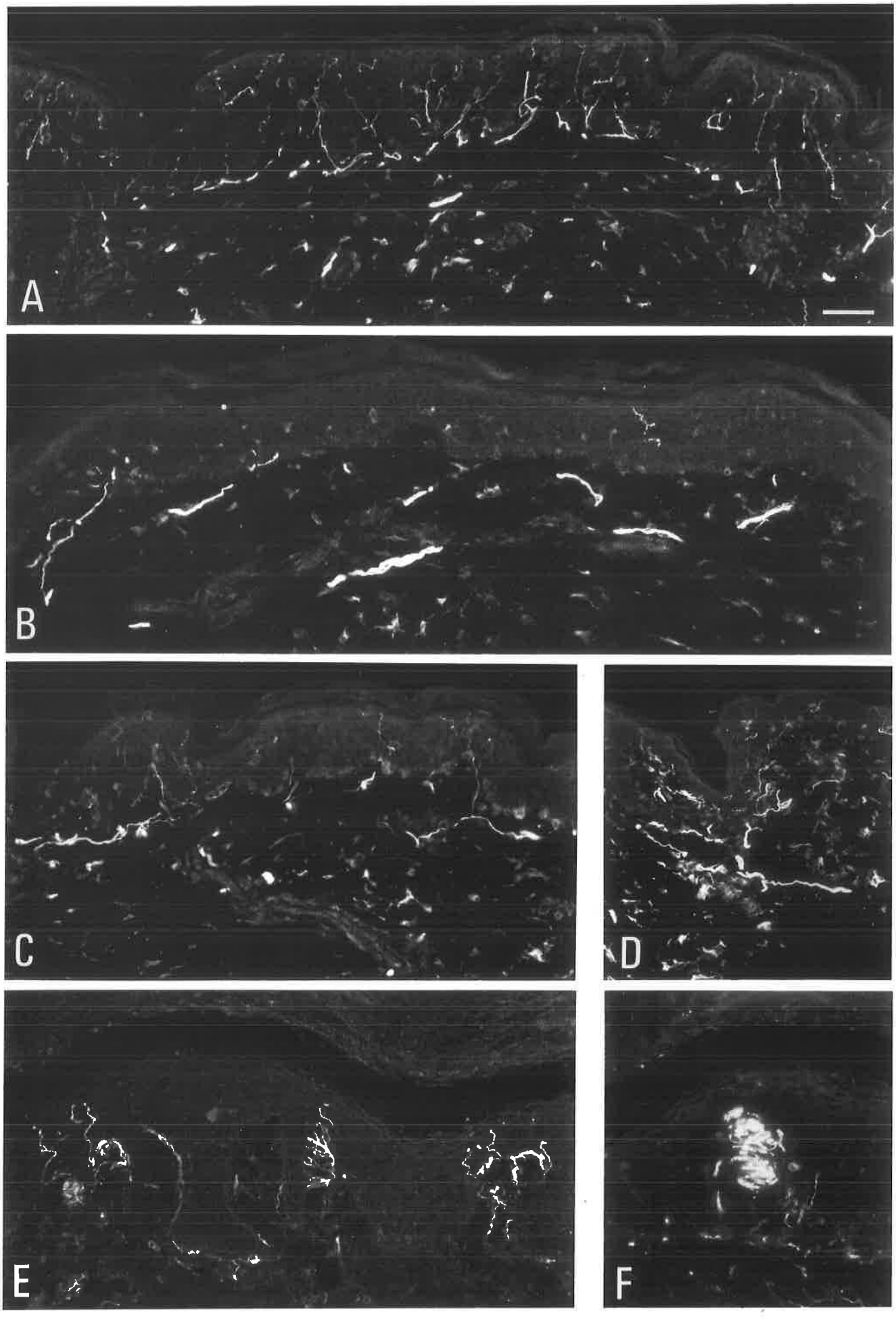
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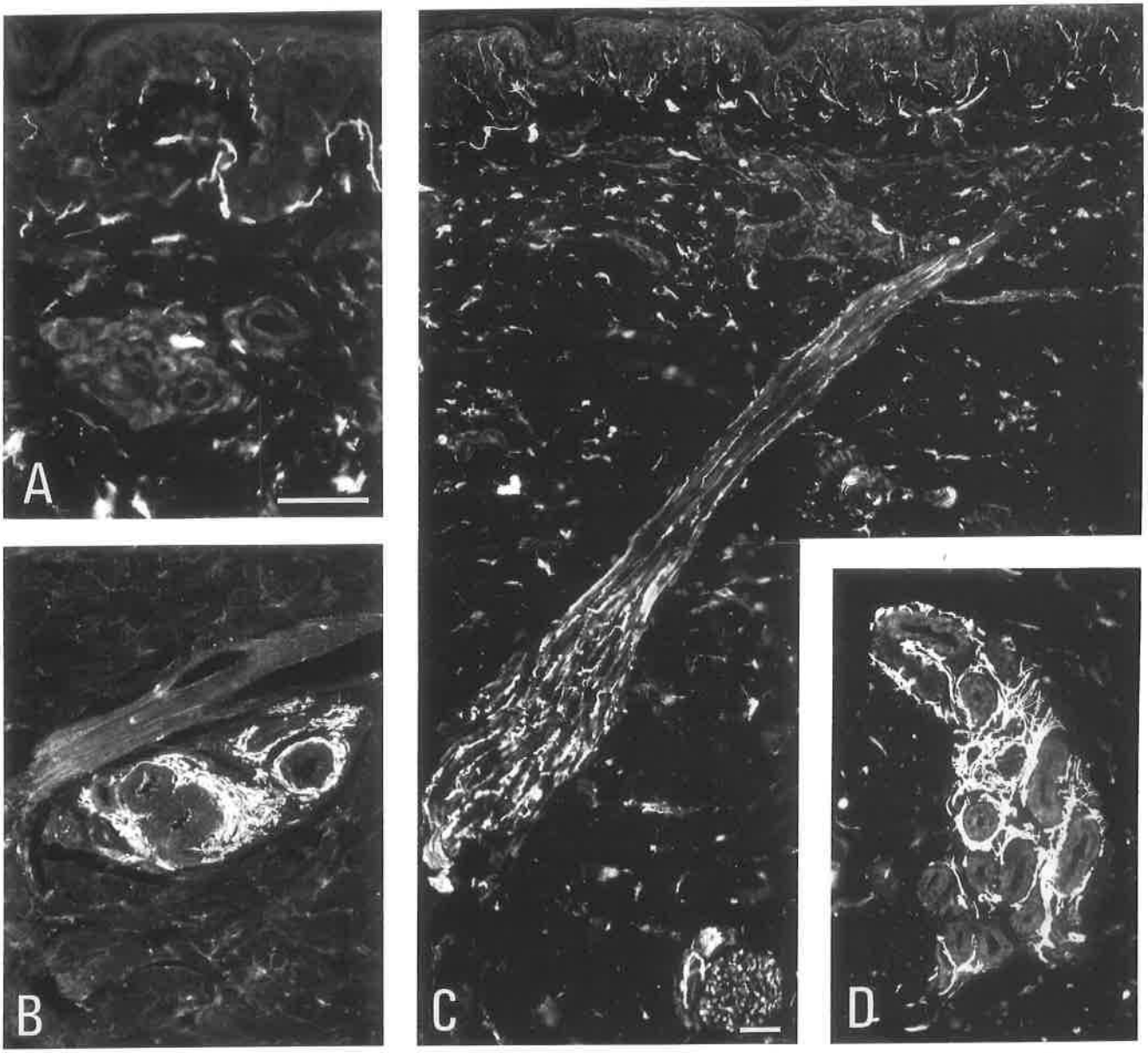
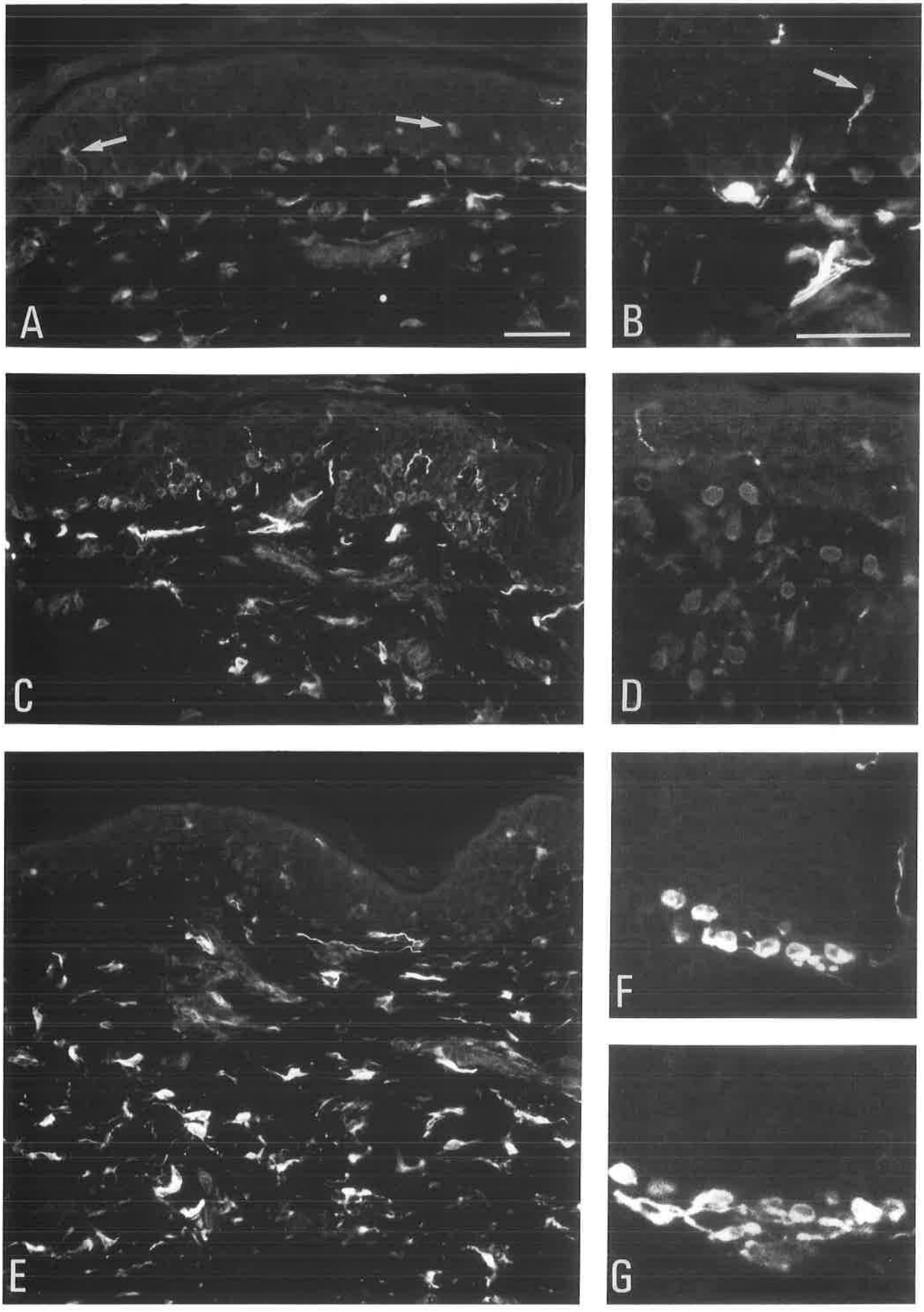


Fig. 3A–D. Immunofluorescence micrographs of human skin showing PGP 9.5-immunoreactive innervation of the blood vessels in the upper dermis of back (**A**) and palm (**B**), muscles of arrector pili (**C**; from back), and the eccrine sweat glands (**D**; from back). Same magnification as in **A** and **B** or **C** and **D**, respectively. *Bars*: 50 μm

Cells

Numerous cells displaying PGP 9.5 immunoreactivity were found both in the epidermis and dermis (Fig. 4). The majority of the epidermal PGP 9.5-positive cells were distributed unevenly in cell clusters within the stratum basale, having an oval or round cellular profile and being weakly fluorescent; however, a few cells exhibited a strong immunoreactivity. Some of the cell clusters resembled Merkel cells, some melanocytes. Occasionally, a few cells with processes (dendritic cells of the Langerhans' type?) were revealed with PGP 9.5 in the stratum spinosum (cf. Fig. 4A). The same cellular shapes could be seen in the upper dermis, especially in the dermal papillae, and in the epidermal-dermal junctional area (Fig. 4A–D). The distribution of these cells differed from area to area; however, many PGP 9.5-positive dendritic cells were scattered in the dermis, and they were heterogenous in form, such as triangular, spindle-shaped or oval, with a large range of vertical projections. The

Fig. 2A–F. PGP 9.5-immunoreactive nerves in the upper dermis of human skin (indirect immunofluorescence technique). **A** Back; **B** lower leg; **C** upper arm; **D** chest; **E** palm; **F** Meissner's corpuscle in palmar dermal papilla. *Bar*: 50 μm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immunofluorescence of these latter cells was stronger than that of the epidermal elements.

In the finger-tip and palm, PGP 9.5-positive cells were much less frequent compared to the other cutaneous areas. Immunoreactive cells were observed at the bottom of the epidermal ridges; few, if any, occurred in the dermis. Those located in the epidermis were small and oval with their long axis parallel to the basal membrane; they closely resembled Merkel cells (cf. Fig. 4F, G).

Discussion

Doran et al. (1983) were able to isolate and characterise PGP 9.5 using two-dimensional electrophoresis, and thereafter raise rabbit anti-human PGP 9.5 antiserum. Using immunoperoxidase labelling they localised PGP 9.5 to neurones in the human cerebral cortex with no evidence of staining of glial elements. PGP 9.5 was estimated to be present in brain at concentrations of 200–500 μg/g wet weight and represent a major protein component of neuronal cytoplasm. Doran et al. (1983) suggested this neurone-specific cytoplasmic marker to be useful in studies of neuronal development and for the detection of neuronal damage in diseases of the nervous system.

Various subpopulations of autonomic and sensory nerves supplying e.g., the mammalian cardiovascular system have been demonstrated by means of specific immunocytochemical and histochemical methods; however, no single marker has previously been available for the visualisation of the entire innervation. Gulbenkian et al. (1987) and Wharton et al. (1988) used PGP 9.5 as a general marker for such mixed arrays of nerve fibres, and PGP 9.5 proved to be a useful substance for examination of regional variations in cardiovascular innervation and determination of the relative proportions of nerve subpopulations. Thompson et al. (1983) reported the presence of cytoplasmic PGP 9.5 in the human DNES and demonstrated PGP 9.5 in large amounts in neurones of human central and peripheral nervous systems. The presence of PGP 9.5 paralleled that of NSE. Staining for PGP 9.5 was displayed in central and peripheral neurones and axons, in scattered anterior pituitary cells, melanocytes of the skin, thyroid parafollicular cells, pancreatic islets, and also in gastric enteroendocrine cells and adrenal medullary cells. Since PGP 9.5 does not represent any previously described brain-specific protein (Doran et al. 1983) and does not appear to correspond to any characterised neurotransmitter enzyme (McGeer et al. 1978), the authors concluded (i) that it is a general marker for neurones and elements of the DNES, and (ii) that it is entirely distinct from NSE. However, it should be noted that the two proteins (PGP 9.5; NSE) normally co-localise immunohistologically, with some exceptions.

Convincing evidence that free nerve endings exist in the epidermis of human skin has been obtained with the neurone-specific marker PGP 9.5 in this study. The distribution and configuration of these intraepidermal nerves are similar to the respective features of silver-impregnated and methylene blue-stained nerve fibres (Arthur and Shelley 1959; Novotny and Gommert-Novotny 1988). Previously, nerves containing neuroactive compounds have been demonstrated in human epidermis, e.g., elements immunoreactive for neurone-specific enolase (NSE), neurofilament (NF), substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A (NKA), somatostatin, and galanin (see Dalsgaard et al. 1983, 1984, 1989a, b; Björklund et al. 1986; Gibbins et al. 1987; Johansson 1987; Johansson and Vaalasti 1987; Johansson et al. 1988, 1989a, b; Vaalasti et al. 1988; Weihe and Hartschuh 1988); however, all these fibres were rare and appeared mostly in the basal layer of the epidermis.

In rat and cat snout epidermis, CGRP- and SP-immunoreactive nerve fibres reach the stratum lucidum; they are more dense and straight than the corresponding elements in human epidermis (Alvarez et al. 1988; Kruger et al. 1989). Such variations may result from species differences or from different affinities of antisera (and also reflect different functional requirements).

Munger and Ide (1988) proposed that axons enter the epidermis either to terminate as free nerve endings or to become associated with Merkel cells. We have not gained persuasive evidence for this pattern, but the intraepidermal PGP 9.5-positive nerve endings *do* terminate in the basal layer as well as in the superficial layers with different shapes and directions; whether they establish contacts with Merkel cells in the basal layer or with keratinocytes, melanocytes, etc., requires additional electron-microscopic investigation.

Certain previous studies have shown that the intraepidermal nerves end with a distinct 'knob' (Munger 1965; Novotny and Gommert-Novotny 1988; Kruger et al. 1989). However, in our material, we observed that the PGP 9.5-containing nerve fibres only occasionally form a corresponding type of terminal 'knob' (cf. Fig. 1B) or preterminal swelling. This calls for further confocal fluorescence imaging and ultrastructural investigation, which are already in progress.

The distribution and density of the nerve terminals in the epidermis displayed by PGP 9.5 immunofluorescence have raised several questions regarding the function(s) of the intraepidermal nerves and the relationship between receptors and sensory modalities. Are they associated with nociception as traditionally conceived, since they are free nerve endings? Why do humans have more intraepidermal nerves on the back than in the hand?

Fig. 4A–G. PGP 9.5-immunoreactive cells in human skin (indirect immunofluorescence technique). **A–C** Round or oval-shaped cells in the stratum basale (**A** and **B** from lower leg; **C** from upper arm). Note strongly immunofluorescent cell in **B. D** Round or oval-shaped cells in the epidermal-dermal junctional area (from chest). **E** Dendritic cells in the dermis (from chest). Dendritic cells also are present in the stratum spinosum (*arrows* in **A** and **B**). **F, G** Merkel-cell clusters in the palm. Same magnification as in **A, C, E** or **B, D, F, G**, respectively. *Bars*: 50 μm

Perhaps pain perception is less important in more mobile and readily contacted parts of the body. Furthermore, the possibility of neuronal trophic interactions in the epidermis cannot be excluded. Finally, these nerves may convey signals to the central nervous system concerning immune functions, since the skin is the main interface between the organism and the environment and it, thus, possesses numerous immune cells (cf. Longley et al. 1988; see also Weihe and Hartschuh 1988).

The PGP 9.5-positive cells are supposed to belong to several cell groups: (1) *Merkel cells* can be clearly identified in the fingertip and palm where they are located at the bottom of the epidermal ridges; they are small and oval with their long axis parallel to the basal membrane. (2) *Melanocytes* are unevenly distributed in the stratum basale; they are numerous in some regions of the body and in some persons, but only few occur in the glabrous skin. (3) *Dendritic cells* are mostly observed in the stratum spinosum and upper dermis where they exhibit a typical morphology. This group is complex, consisting of several cell types, e.g., Langerhans cells.

All epidermal and dermal nerve fibres and also certain cutaneous cells display brilliant immunofluorescence when PGP 9.5 is used as a marker substance. Judged from the present data, PGP 9.5 is a *total* marker for the nervous structures in the epidermis and dermis; however, there is still a possibility that not all the nervous elements of the human skin have been demonstrated. At least we are unable to find PGP 9.5-labelled nerves around some small blood vessels (cf. Fig. 3A), although previous investigators have succeeded by use of PGP 9.5 antiserum in demonstration of the entire innervation in the cardiovascular and uterine systems of the guinea pig (cf. Gulbenkian et al. 1987; Lundberg et al. 1988). In this context, it may also be noted that Osborne and Neuhoff (1985) missed specific immunofluorescence in certain neuronal components of the rabbit retina. The reason for this differential reactivity pattern remains unknown. In addition, the biological function of PGP 9.5 is still a matter of debate.

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