Journal of Neurocytology **23**, 577–589 (1994)

Substance P, neurofilament, peripherin and SSEA4 immunocytochemistry of human dorsal root ganglion neurons obtained from post-mortem tissue: a quantitative morphometric analysis

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Received 23 December 1993; revised 25 April 1994; accepted 3 May 1994

Summary

Immunocytochemical studies on lumbar dorsal root ganglia obtained at routine postmortem 24–36 h after death were carried out, and neuronal cross-sectional areas measured. The subjects were elderly (76–81 years), of both sexes, had died from heart attack or haemorrhage, and had no clinical evidence of clinical neuropathy or of disease known to be associated with neuropathy. The data were consistent between ganglia from the three subjects. There were striking similarities with data from other species. Two populations of cell profiles with overlapping size distributions were distinguished with an anti-neurofilament antibody, neurofilament-rich (45% of cell profiles) with a large mean area and neurofilament-poor with a smaller mean area. Anti-substance P and anti-peripherin antibodies both labelled a population with a small mean area, with extensive co-localization between them. There were also some differences between these human dorsal root ganglia and dorsal root ganglia from some other species. More neuronal profiles were labelled for substance P in humans (44%) than in rat (20%). More neuronal profiles were labelled for SSEA4 (stage specific embryonic antigen 4) in human (40.5%) than in rat dorsal root ganglia (10%), and the SSEA4-positive profiles were relatively smaller in human than in rat. No selective accumulation of lipofusin in profiles of large cells was apparent. This study also shows that quantitative morphometric analysis of immunocytochemically labelled dorsal root ganglion neuronal profiles can be carried out successfully on human sensory ganglia obtained at post-mortem. This is the first demonstration of the two main subgroups of dorsal root ganglia neurones with neurofilament-rich and poor somata in human tissue. The size distributions of neurons with neurofilament, substance P and peripherin are consistent with these neuronal populations having similar functional properties to those described in other species. From the known sensory and fibre loss with aging, it is speculated that the loss of some large diameter neurones with myelinated fibres and low mechanical thresholds, might account for the high percentage of neurones expressing substance P.

Introduction

A number of markers such as neurofilament (Schlaepfer & Lynch, 1977; Horie *et al.*, 1989; Suburo *et al.*, 1992), substance P (SP) (Charnay *et al.*, 1983; Pioro *et al.*, 1984; Suburo *et al.*, 1992), calcitonin gene-related peptide (CGRP) (Gibson *et al.*, 1984; Suburo *et al.*, 1992), low affinity nerve growth factor (NGF) receptor (Sobue *et al.*, 1989; Suburo *et al.*, 1992) and certain glycoconjugates (Kusunoki *et al.*, 1991), have previously been shown to label foetal or adult human sensory neurones. However, published studies on quantitative information on the distribution of these markers in human ganglia are few (e.g. Sobue *et al.*, 1989; Del Fiacco *et al.*, 1990; Suburo *et al.*, 1992; Quartu *et al.*, 1992). Such studies are needed to establish the extent of similarity between animal (see review by Lawson, 1992) and human ganglia. They would also allow an evaluation of whether selective groups of sensory neurons are lost with age or in diseases known to be associated with peripheral neuropathy, such as carcinoma, renal failure and diabetes. We also wished to establish the extent to which post-mortem tissue was immunoreactive to these antibody markers and the variability between ganglia derived from subjects of similar age with 'normal' ganglia, that is in subjects who have died from causes which have no known neuropathy associated with them. To study

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0300–4864/94

this, we have made a quantitative examination of dorsal root ganglia (DRG) from elderly human subjects using four immunocytochemical markers for subsets of sensory neurons in other species (Lawson, 1992).

The distributions of two types of intermediate filaments were examined. Neurofilaments were examined because in rat, cat and quail DRGs the two main neuronal types, known in the rat as the 'large light' or A neurons and the 'small dark' or B neurons have, respectively, neurofilament-rich and neurofilament-poor neuronal somata (Lawson *et al.*, 1984; Perry *et al.*, 1991; Lawson *et al.*, 1993; Perry & Lawson, 1993). In the rat these populations give rise, respectively to myelinated and unmyelinated fibres (Lawson & Waddell, 1991). The antibody known as NFH was used because it was very effective at labelling the whole of the large light cell population in both cat and rat DRGs (Perry *et al.*, 1991; Perry & Lawson, 1993). We have therefore investigated whether these two main types of sensory neuron are identifiable in human DRGs.

The second type of intermediate filament protein that we examined was peripherin which has an almost reciprocal labelling pattern with neurofilament in rat and mouse DRGs (Ferri *et al.*, 1990; Troy *et al.*, 1990). We have therefore examined whether this is also likely to be the case in human DRGs.

Substance P-like immunoreactivity (SP-LI) was examined because this peptide is one of the most widely studied peptides in DRGs of other species, and because there is considerable information about its function. Substance P release from the peripheral terminals of primary afferents is thought to be involved in the neurogenic inflammatory response e.g. (Holzer, 1988; Nagahisa *et al.*, 1992; Xu *et al.*, 1992). It is released from central terminals of DRG neurons in response to noxious mechanical and in some cases noxious heat stimuli (Kuraishi *et al.*, 1985; Duggan *et al.*, 1988). Recent studies have shown a clear correlation between SP-LI in DRG neuronal somata of the guinea-pig and the cutaneous sensory receptor type (Lawson *et al.*, 1994). It is in about 20% of rat lumbar DRG neurons with a small mean diameter (McCarthy & Lawson, 1989; Lawson, 1992). In the rat, a third of somata with SP-LI were neurofilament-rich and two thirds were neurofilament-poor (McCarthy & Lawson, 1989; Lawson *et al.*, 1993). Since in rat DRGs the majority of neurofilament-poor (small dark) neuronal somata express peripherin (Ferri *et al.*, 1990), and since the majority of SP-LI neurons are small dark (Lawson *et al.*, 1993), there should be extensive co-localization between these markers. This we have tested. Because of its functional importance and the present pharmaceutical interest in tachykinins and their receptors, it is important to establish whether the neuronal population expressing SP in human DRGs resembles those in the well studied species such as rat and guinea-pig.

The fourth antibody, stage specific embryonic antigen 4 (SSEA4), was chosen as an example of a cell surface carbohydrate marker and also as the only such marker which so far has been related to sensory function in experimental animals. Stage specific embryonic antigen 4, is a globoseries carbohydrate antigen on a surface glycolipid. In guinea-pig it is expressed in some DRG neurones with high threshold mechanoreceptor (i.e. nociceptor) properties (Perl, 1991). Since SSEA4 is expressed in medium to large neurons (large light neurons) in rat and guinea-pig (Dodd *et al.*, 1984) and personal observations, we have tested whether SSEA4 is expressed in neurofilament-rich somata in human DRG neurons.

Lipofuscin accumulation is the clearest change known to occur in aging DRG neuronal somata. This is cellular debris partly derived from free-radical induced peroxidation of cellular constituents. In rat there is evidence that it accumulates earlier in the large light (i.e. neurofilament-rich) DRG neuronal somata than in the small dark (i.e. neurofilament-poor) neuronal somata (Van den Bosch de Aguilar & Vanneste, 1981). We have tested whether this is also the case in human DRGs.

Some of this data has been published in abstract form (Holford *et al.*, 1993; Case *et al.*, 1994).

Materials and methods

Source of tissue

Human lumbar L5 DRGs obtained during routine post-mortem examinations were obtained from the following three subjects; a 78-year-old male: cause of death retroperitoneal haemorrhage; a 76-year-old male and an 81-year-old female: cause of death heart attack. The clinical notes and post-mortem findings were checked to ensure that there was neither clinical evidence of peripheral neuropathy nor any evidence of malignancy, diabetes, renal failure or drugs known to cause peripheral neuropathy. The ganglia were removed at 24–36 h post-mortem, were sliced longitudinally to improve fixation and placed in Zamboni's fixative at 4° C for 4 h. They were then left overnight in 30% sucrose in 0.1 M phosphate buffer at 4° C. The tissue was then mounted in Tissuetek and several series of 10 μm frozen sections each with sections spaced at 1 mm intervals. They were stored at −20° C.

Immunocytochemistry

Double labelling indirect immunofluorescence immunocytochemistry was used. Each series measured was incubated at 4° C for 48 h with two primary antibodies in a solution containing 0.1 M phosphate buffered saline (PBS) containing 10% foetal calf serum, 0.2% Triton X-100 and 1% normal goat serum. The pair of primary antibodies used was either NFH (1:200) and SSEA4 (1/150) or anti-peripherin (1:200) with anti-SP (1:50). For details of antibodies see below.

The sections were washed with PBS (3 × 10 mins),

incubated for 30 min at room temperature with the second layer antibodies conjugated to fluorescent labels (see below). The sections were washed with PBS containing 1% normal goat serum and 0.5% Triton X-100 (2 × 10 min) and with PBS with 1% normal goat serum (1 × 10 min). The slides were mounted in carbonate buffered glycerol.

To investigate the specificity of the SP antibody, immunocytochemistry was also carried out with antibodies which had been pre-absorbed with SP (1×10^{-6} M) for 1 h at room temperature.

Materials

Primary antibodies were NFH (Breen *et al.*, 1988) (rabbit polyclonal), a gift from B. Anderton; SSEA4 (Dodd *et al.*, 1984) (mouse monoclonal antibody to SSEA4), a gift from P. Goodfellow; anti-peripherin (Ferri *et al.*, 1990) (rabbit polyclonal), a gift from M. Portier, and anti-SP (rat monoclonal, Seralab).

Secondary antibodies were goat anti-rabbit IgG, conjugated with AMCA (7-amino-4-methyl coumarin-3-acetic acid) for NFH and anti-peripherin, and goat anti-mouse IgG conjugated to FITC (fluorescein isothiocyanate) for SSEA4 and SP.

Measurement of neuronal profiles

The sections were examined with a 40 × objective on a Leitz Dialux 22 microscope. Areas of neuronal profiles with clearly visible nuclei were measured by drawing their outlines under interference contrast optics with a Graf pen interfaced to a microcomputer.

By switching to epifluorescence optics, the labelling with the two fluorochromes, as well as the approximate area of the cell profile occupied by lipofuscin, could be quantitated. The AMCA and FITC were visualized using the A and I2 filters respectively. Immunoreactivity of the cells to the markers were graded on a 0–5 scale as a subjective measure of the intensity of the fluorescence, with 0 being negative and 1–5 positive (5 as most intense). Lipofuscin was visible through both of the above filters, although with a very obvious colour shift towards the orange compared with the blue (AMCA) or green (FITC) colours of the fluorochromes. It was thus easily distinguishable from both these fluorochromes. Its extent could also be assessed using the N2 filter, through which it appeared red. The amount of lipofuscin in a cell profile was graded using a similar 0–5 scale, 0 being no lipofuscin visible, and 1–5 representing the subjectively judged proportion of the cytoplasmic area filled with lipfuscin (1:0–20%; 2:20–40%; 3:40–60%; 4:60–80%; 5:80–100%). This information was fed into the computer with the size of the profile, and the computer was used to plot histograms of cell cross-sectional area in relation to the fluorescent labelling patterns.

Results

General immunocytochemical staining of tissue

Good immunocytochemical labelling was achieved with all four markers used. Each marker distinguished two populations of cell profiles; those that were clearly labelled by the marker and those that were poorly labelled or unlabelled by the marker. A very small percentage of cell profiles (usually about 1–3%) fell at the borderline of these two classifications. The appearance of the cells is shown in Fig. 1. It can be seen that there is a range of intensities of labelling for each of the markers used, but that virtually all cell profiles could be placed into one of these two populations. Lipofuscin could be clearly visualized both under interference contrast, under which it appeared brown and granular, and under fluorescence illumination when it appeared as a bright granular substance through all filter blocks (Fig. 1C).

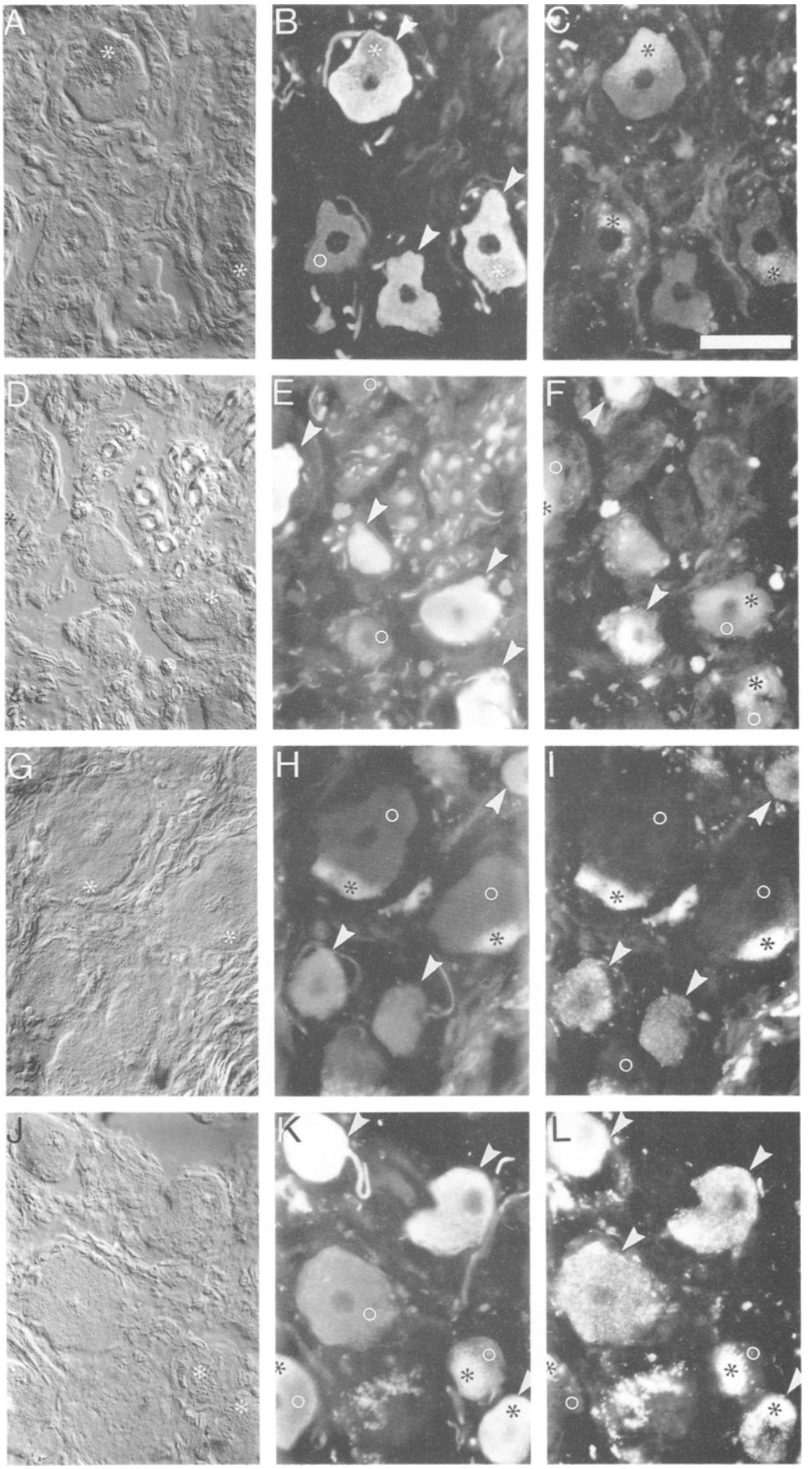
Neurofilament staining

NFH clearly distinguished two populations of cell profiles (Fig. 1B,E). One population was intensely labelled by NFH and was designated neurofilament-rich. This population included 45% of all cell profiles, had a large mean area (2488 μm^2) and included virtually all cell profiles with an area of >2000 μm^2 plus some smaller neurons (Table 1). The other population (designated neurofilament-poor) showed much less intense labelling. This population (mean area 1398 μm^2) contained most small cell profiles. The distributions of cross-sectional areas for neurofilament-rich and neurofilament-poor cell populations is shown in Fig. 2A. The size distributions of the two populations overlapped to a considerable extent.

Stage specific embryonic antigen 4

Cells clearly positive for SSEA4 (40.5% of all cells, mean area 1409 μm^2 (Table 1)) had a smaller mean area than those which were negative (that is unlabelled or very lightly labelled) (mean area 2208 μm^2), see Figs 1F, 2B. The positive cells were confined to the small to

Fig. 1. Photomicrographs of human lumbar DRG neurons. Each row of photographs shows the same field of a section. (A–C) A interference contrast; B NFH (AMCA); C lipofuscin autofluorescence; (D–F) D interference contrast; E NFH (AMCA); F SSEA4 (FITC); (G–I) G interference contrast; H peripherin (AMCA); I SP (FITC); (J–L) J interference contrast; K peripherin (AMCA); L SP (FITC). Fluorochrome given in parentheses; in C the filter for FITC was used. Arrowheads indicate examples of positive labelling; open circles indicate examples of negative or neurofilament-poor labelling; asterisks indicate areas in cells with lipofuscin where visible due to its granularity or brown pigment under interference contrast, or its autofluorescence through either filter block. Note the absence of neurofilament immunoreactivity in regions of the cell with abundant lipofuscin (B). Scale = 50 μm.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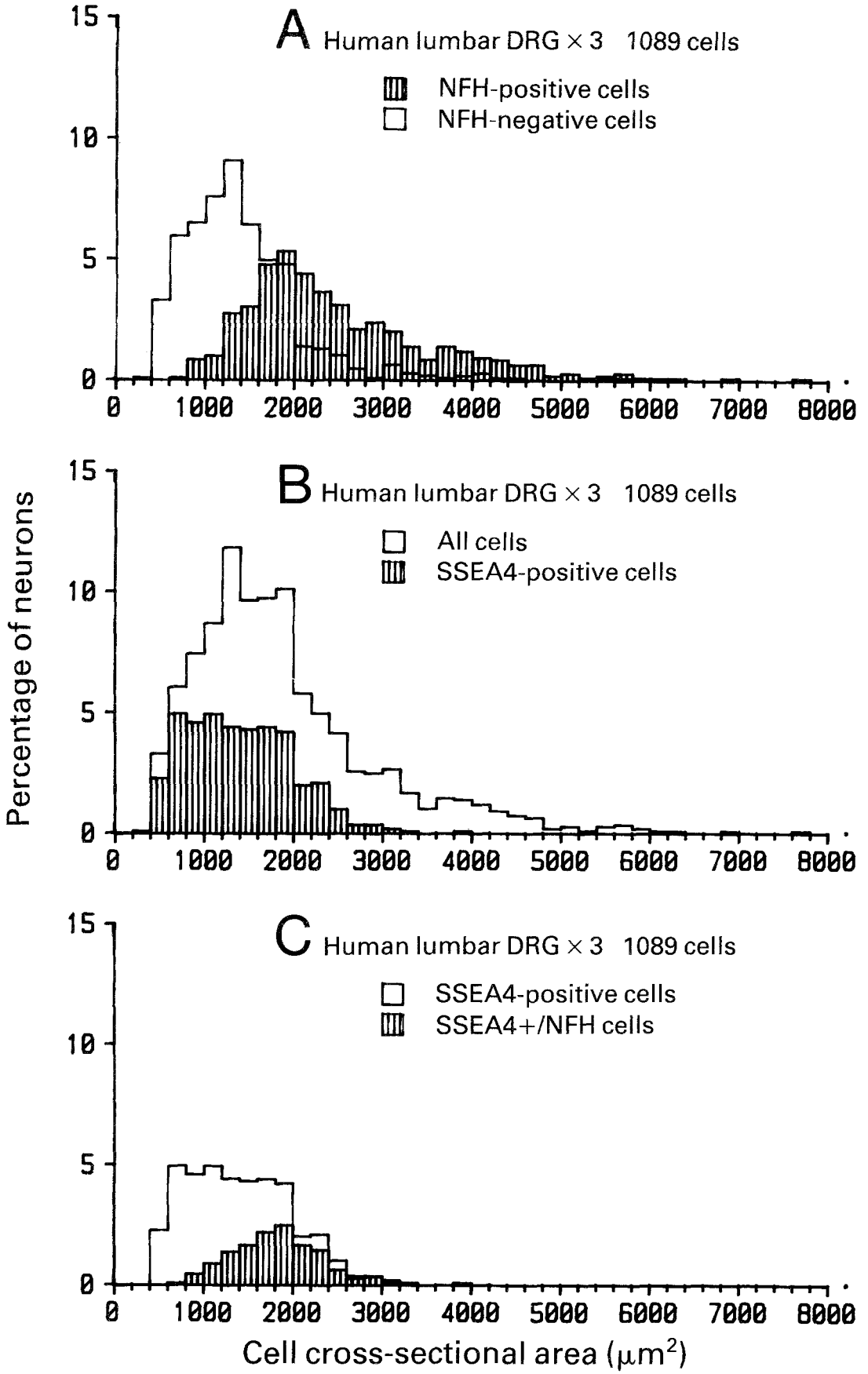

Fig. 2. Histograms of cross-sectional areas of neuronal profiles measured at the nuclear level to show pooled data from lumbar DRGs of the three subjects. (A) Two populations of cell profiles distinguished as neurofilament-rich (NFH-positive cells) or neurofilament-poor (NFH-negative cells). (B) The size distribution of neuronal profiles labelled for SSEA4, vertical hatching, and the overall size histogram for the cell profiles in A, B and C (open histogram). (C) The subpopulation of SSEA4-positive cell profiles (open histogram) also labelled by NFH, vertical hatching.

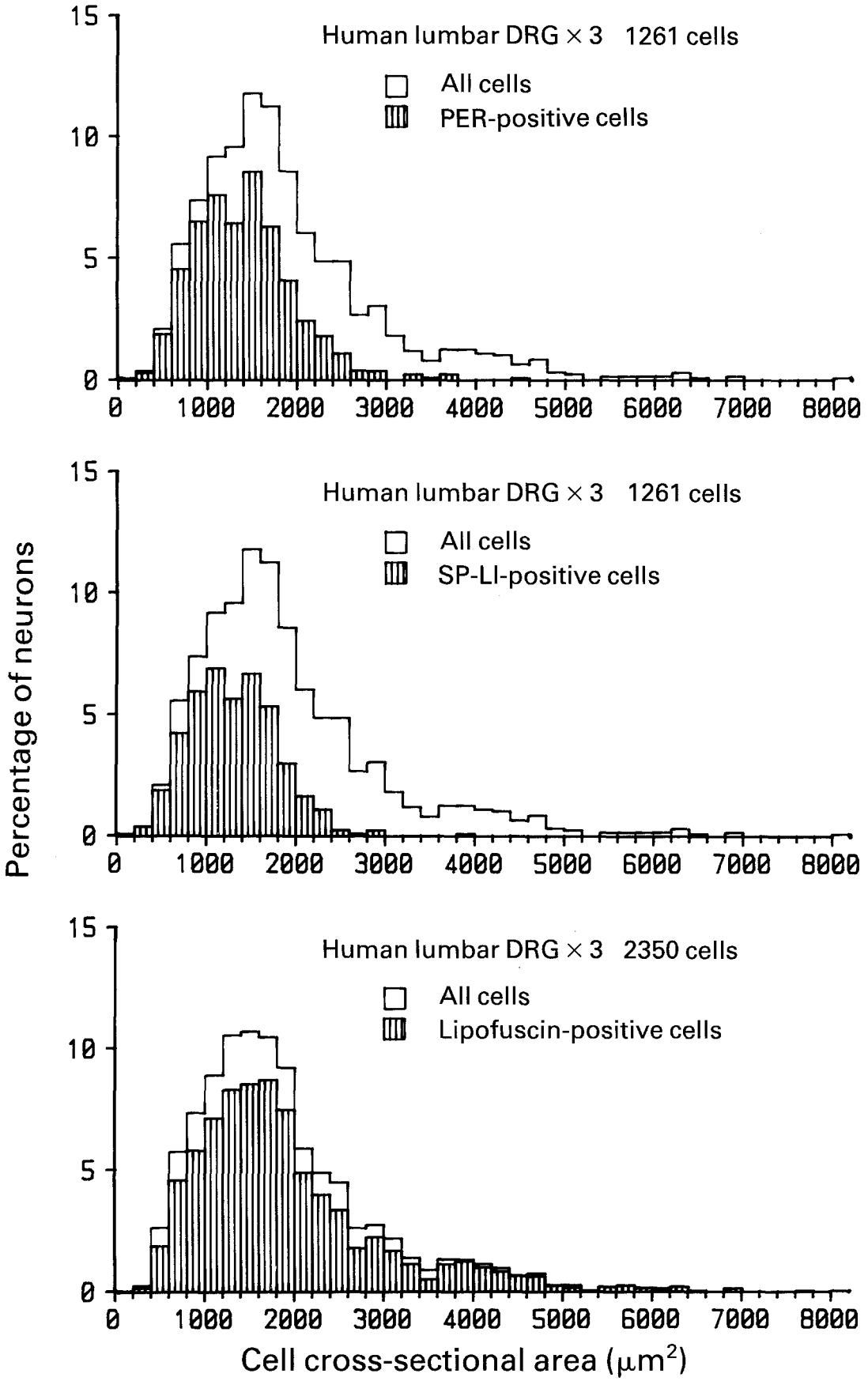

Fig. 3. Histograms of cross-sectional areas of cell profiles measured at the nuclear level to show: (A) the size distribution of profiles labelled for peripherin (PER) (vertical hatching) and all cell profiles measured (open histogram). (B) the size distribution of profiles with SP-LI (vertical hatching) and all cell profiles (open histogram). (C) Overall size distribution of cell profiles from the DRGs from the three subjects (open histogram) and of those with some visible lipofuscin within them (vertically hatched histogram). All cell profiles in all the series counted for NFH/SSEA4 and for SP/peripherin from the three subjects are included in this histogram.

Table 1. Percentages and mean areas of cells labelled by the different markers

		Area (μm^2)		
	% of cells	*Mean*		*SD*
All cells	100	1888	±	1041
NFH high	44.9	2488	±	1063
NFH low	55.1	1398	±	709
SSEA4-positive	40.5	1409	±	591
SSEA4-negative	59.5	2208	±	114
SP-positive	44.1	1306	±	489
SP-negative	55.9	2353	±	1134
PER-positive	54	1405	±	567
PER-negative	46	2455	±	1178

The total number of cells counted in each case is given on the histograms (Figs 2, 3). % is the percentage of the total cells counted in each case. PER is peripherin. Since the areas of all cells were measured on two series (that for SP/PER and that for NFH/SSEA4) the mean area and SD for all cells given here result from adding these values from the two series and dividing by two.

medium end of the overall size range. The SSEA4-positive population had a similar size range to that of the neurofilament-poor population (Table 1) and indeed most (66%) of the SSEA4-positive cells were neurofilament-poor, (see Fig. 2C).

Peripherin

The neurons labelled for peripherin (54% of all cell profiles, mean area 1405 μm^2, Table 1) contained most small cells and corresponded in size distribution to the neurofilament-poor population. The neurons which were unlabelled for peripherin (mean area 2455 μm^2) contained most large cells and corresponded in size distribution to the neurofilament-rich population. Fig. 1H,K shows the appearance of a typical field of view from a section labelled for peripherin, with small positive cells and larger negative cells. Although the absolute intensity of labelling varied from one subject to another (the whole field in Fig. 1K is more intensely labelled than in Fig. 1H) the cells from each subject could easily be designated as clearly labelled or weakly/unlabelled. Figure 3A shows the distribution of the cross-sectional areas of the peripherin-positive neuronal population compared to that of the total population.

Substance P

The population of neurons with SP-LI (44.1% of all cell profiles, mean area 1306 μm^2, Table 1) contained relatively small cell profiles. It corresponded (see Fig. 3B) in size range to both the neurofilament-poor population and the peripherin-positive population. Figure 1I and L shows the characteristic granular appearance in this tissue of the SP-LI staining.

Immunocytochemistry with antibody which had been pre-absorbed with SP resulted in no SP-LI staining in neurons or fibres within the sections of DRG.

Lipofuscin

Cross-sectional areas of cell profiles which contained lipofuscin (80.5% of all cells) and of cells which contained no lipofuscin (19.5% of all cells, Table 1) are shown in Fig. 3C. Data are from all cells counted for all four markers. The size ranges of the neurons with and without lipofuscin are extremely similar. The areas of cell profiles with gradings 1, 2, 3, 4 and 5 (for the area of cytoplasm filled with lipofuscin) were plotted separately, but in all cases the size distributions were similar, showing no evidence of a selective accumulation of large amounts of lipofuscin in small or large neurons. It can be seen in Fig. 1C that the lipofuscin in the upper large cell occupies the cytoplasm on one side of the cell, and, perhaps because of this volume occupied, in this region there is less anti-neurofilament immunoreactivity.

Co-localization of labelling

Table 2 shows the co-localization between the staining with the pairs of antibodies. It also shows the co-localization between lipofuscin and the antibodies used. There was extensive colocalization between SP-LI and peripherin labelling; 92% of neuronal profiles with SP-LI were also peripherin-positive and 75.3% of peripherin-positive profiles also showed

Table 2. Colocalization of labelling

	Lipo-fuscin	*NFH*	*SSEA4*	*PER*	*SP*
Lipofuscin-positive	–	44	41	57*	46*
-negative	–	48	39.5	37*	34*
NFH-positive	75	–	31*	–	–
-negative	78	–	48*	–	–
SSEA4-positive	77	34.3*	–	–	–
-negative	76	52*	–	–	–
PER-positive	89*	–	–	–	75.3*
-negative	77*	–	–	–	7.5*
SP-positive	87*	–	–	92*	–
-negative	81*	–	–	24*	–

Percentages of DRG neuronal populations labelled by markers in column 1 (100%) that are also labelled by markers in row 1; e.g. 46% of lipofuscin-positive cells showed SP-LI, but only 34% of lipofuscin-negative cells showed SP-LI. 2 × 2 contingency tables and Fisher's exact significance test showed whether proportions of labelled cells arose by chance. There was no significant association between lipofuscin presence and either NFH labelling ($p = 0.35$) or SSEA4 labelling ($p = 0.71$). The greater proportion of lipofuscin presence in neurons with SP-LI or peripherin (PER) than in those without did not arise by chance ($p = <0.00001$ in both cases). Similarly, tests for association between the two pairs of antibodies used showed that the strong association between SP-LI and peripherin did not arise by chance ($p < 0.00001$). Asterisks indicate values which show highly significant associations.

SP-LI. 2 × 2 Contingency tables and Fisher's exact significance test showed a significant association between SP-LI and peripherin labelling ($p < 0.00001$). The colocalization between the peripherin labelling and SP-LI labelled populations is shown in Fig. 3C. The population which showed no SP-LI contained mainly larger cells and had a similar size distribution to that of the peripherin-negative population. In contrast, colocalization was much less extensive between NFH and SSEA4, (about a third of neurofilament-positive cell profiles were labelled for SSEA4 and vice versa).

A significantly higher proportion of neuronal profiles with lipofuscin than of those without showed peripherin and SP labelling ($p < 0.00001$, 2 × 2 contingency tables and Fisher's exact significance test). No such association was seen between lipofuscin and NFH or SSEA4 labelling.

Consistency of data between subjects

Figure 4 shows the distribution of cross-sectional areas of neuronal profiles with SP-LI compared to that of the total population for each of the three subjects. It can be seen that although there are differences in the proportion of labelled profiles in each subject (see Table 3), the size distribution of the profiles with SP-LI is consistent for all three subjects and the mean areas of the populations with SP-LI in the three individual ganglia were very close (see Table 3). For the other markers, both the percentages and the mean size of the positive cell profiles showed very good consistency between subjects (Table 3).

Table 3. Comparison of data from DRGs of the three different individuals (A, B and C) to show the variability

		Labelled			*Unlabelled*		
		%	*Mean area*	*SD*	%	*Mean area*	*SD*
NFH	A	48	2368	±849	51	1268	445
	B	43	2618	±1377	56	1702	994
	C	40	2549	±995	57	1275	552
SSEA4	A	39	1342	±565	60	2106	900
	B	37	1652	±579	60	2368	1482
	C	43	1295	±578	55	2191	1060
PER	A	59.4	1311	±466	40	2364	929
	B	51	1420	±618	46	2826	1400
	C	47	1558	±635	52	2173	1080
SP	A	56	1297	±471	44	2292	924
	B	40	1290	±465	57	2673	1361
	C	30	1353	±567	69	2102	990

Discussion

One of the reasons for carrying out this study was to establish whether this type of quantitative morphometric analysis combined with immunocytochemistry was feasible on human post-mortem tissue. A possible problem with using such tissue was that breakdown products might bind differently or inappropriately with the antibodies. This we cannot entirely exclude. However for most of these markers there is reason to believe that the appropriate cells were labelled for the following reasons. Other studies have previously successfully demonstrated neurofilament immunoreactivity and SP-LI in human tissue (see Introduction). In the present study, the clear filamentous pattern of labelling in some cells labelled for peripherin and the similarities between staining in these DRGs and in DRGs of other species (see later) provide a strong indication that the immunoreactivity showed the distribution of the original molecules, (reacting either with them or some breakdown product), in this post-mortem tissue. These similarities with other species include the size distributions relative to all cells in the DRG of cell profiles stained for peripherin, neurofilament and SP. Furthermore, the consistency of the pattern of size distributions from three subjects of the same age, plus this similarity of the patterns of labelling and the size distributions of the cell profiles compared with those from other species (see below) gives us confidence that this method can provide useful data about human DRGs, even 24–36 h post-mortem.

It should be noted that the method of sampling of cell profiles used in this paper has no corrections for bias towards larger profiles. Such a bias is inevitable with this method (Coggeshall, 1992) and will tend to somewhat overestimate the proportions of large cells, since they have larger nuclei and a greater chance of sections passing through the nucleus.

Since NFH recognises a phosphorylation independent epitope on the 200 kDa subunit, the labelling may reflect the total amount of this subunit. The staining pattern indicates that all DRG neuronal somata express the 200 kDa subunit, but that a subpopulation is clearly more intensely labelled. A previous study (Suburo *et al.*, 1992) found that all adult human DRG neurones were labelled with the antibody RT97 which recognises a highly phosphorylated form of the 200 kDa subunit. In contrast we found that RT97 stained some neurons of these adult human DRGs but not as many as were strongly labelled by NFH. It was because of this and because in the cat NFH was more effective than RT97 at differentiating the large light and small dark neurons (see Introduction) that we chose to use NFH in this study. A possible explanation for the findings of Suburo and colleagues (1992) is that in rat RT97 at high concentrations can label all DRG neurons (Lawson *et al.*, 1984; and personal observations) and rather high concentrations were used in that study (1:25) (Suburo *et al.*, 1992).

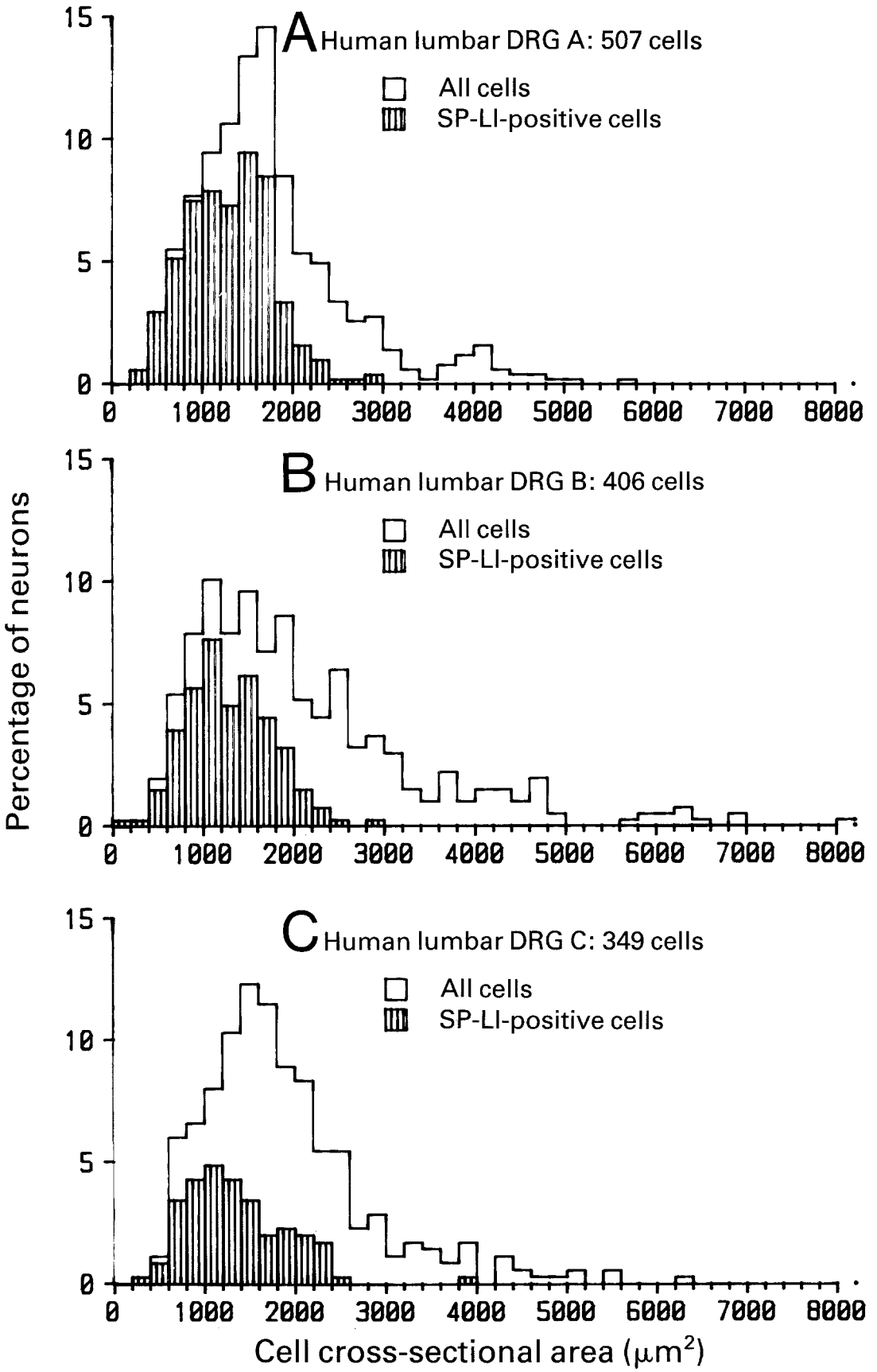

Fig. 4. Histograms of cross-sectional areas of neuronal profiles showing the size distribution of profiles with SP-LI from three individual ganglia. These demonstrate the consistency of area distributions for all cell profiles (open histograms) and SP-LI cell profiles (vertically hatched histograms) between subjects. However, some variability between the proportions of profiles labelled for SP-LI in the different subjects is clear.

Although immunoreactivity against neurofilament subunits in human DRG neurons was previously observed 'especially in perikarya of the large neuronal type' (Schlaepfer & Lynch, 1977), this is the first report that human DRG neurons can be subdivided into the two main populations with neurofilament-rich and neurofilament-poor somata seen in other species. In rat and cat DRGs the neurofilament-rich population have the larger mean area and extend across the entire size range in the ganglion, while the neurofilament-poor cells are limited to the small end of the distribution (Lawson *et al.*, 1984; Perry & Lawson, 1993). The pattern seen in these human subjects resembles this in many ways: the neurofilament-rich population had a wide distribution of cell sizes covering the size range of neurons from nearly the smallest to the largest in the ganglion while the neurofilament-poor neurons formed a population with a more limited size range, consisting of predominantly small cells. Since in rat and cat these populations have been shown to be the same as the light (A neurons) or small dark (B neurons) respectively (from their cytoplasmic staining characteristics with Nissl stains) (Lawson *et al.*, 1984; Perry & Lawson, 1993), a similar nomenclature may be appropriate in the human. In contrast to the rat and cat, however, the two size distributions in human DRGs were skewed, both having a tail to the right. In the case of the neurofilament-rich cells, it is possible that this skew may have resulted from selective loss of larger cells with aging, but further studies are needed to test this hypothesis.

The division of DRG neurons into these two subpopulations on the basis of the labelling of their somatic neurofilament appears, therefore, to be conserved across a number of species, including quail, rat and cat (Lawson *et al.*, 1993) and human. In the rat this division has been shown to be related to the myelination of the peripheral fibre (Lawson & Waddell, 1991), neurofilament-rich somata having myelinated fibres and neurofilament-poor somata generally having unmyelinated fibres. However, it remains to be established whether this relationship is maintained across species. It is likely that the neurofilament-rich neurons in all these species, including human, are those with the fastest conduction velocities on the basis of the rat studies and the larger mean soma size of these neurons in all species studied.

The SSEA4-positive neurons were contained within the peak of small-medium sized cells and comprised 40% of all neuronal profiles. This distribution is very different from that in rat DRG neurons, in which SSEA4 labels the surface of 11% of neuronal profiles, mainly those with an intermediate or large diameter (Dodd *et al.*, 1984; Jessell & Dodd, 1985). It therefore seems that unlike the other three markers in this study, the population of human DRG neurones which express SSEA4 differs in several respects from that in the rat. In the rat, this marker is found on some A fibre neurons with high threshold mechanoreceptive properties (Perl, 1991), but in view of the different relative size distribution of these neurons in human DRGs, it would be dangerous to assume similar receptor properties in SSEA4-positive neurons in the human. Species differences in the distributions in the dorsal horn of primary afferent fibres with oligosaccharide residues (Alvarez *et al.*, 1990) have also been noted previously.

Human DRG neuronal profiles with peripherin-like immunoreactivity show a similar relative size distribution of profiles in rat DRGs (see Ferri *et al.*, 1990; Vickers *et al.*, 1991), in that the majority of small neurons are labelled while few medium or large neurons are labelled. The functional significance of this intermediate filament is not yet clear.

It is likely that the anti-SP antibody is indeed labelling neurons which express SP, from the lack of SP-LI after preabsorption of the antibody with SP and from the finding that the labelled human cells are relatively small, as found for SP-LI in other species. However, we cannot entirely exclude the possibility that there could be non-specific labelling of non-SP related epitopes. The finding of SP-LI in small DRG neurons has previously been noted in the human foetus (Suburo *et al.*, 1992). The similarity of the relative size range in humans and other species e.g. in rat, cat and guinea pig (Lawson, 1992) is consistent with the functional properties of neurons with SP-LI being similar in all these species. The reported percentages (uncorrected) of neuronal profiles with SP-LI in lumbar DRGs in the rat vary from 20–38%, most commonly 20%, and in the cat from 12–29%, (Lawson, 1992). Thus the range of 30–56% in these human DRGs is relatively high. In contrast, a study with the same anti-SP antibody on trigeminal neurons from human subjects aged 67–70 found that only 24% of neurones showed SP-LI (Del Fiacco *et al.*, 1990). In that study cells from only six sections were counted but there was no indication of the number of subjects from which the samples were taken. There are several possible explanations for the higher percentage of neurones with SP-LI compared with other species in the present study (DRGs) but not in the study of (Del Fiacco *et al.*, 1990) (trigeminal ganglion). Firstly, our subjects were all about ten years older and possibly subject to more selective cell death since elevated tactile thresholds in the foot are not clear until ages of over 70 years (Mitchell & Schady, 1988). Secondly, the influences on the aging trigeminal may differ from the DRG since the trigeminal has a dual origin from the neural crest and placode, while the DRG is purely neural crest in origin. The latter may relate to different requirements of neural crest and placodally derived neurons on particular growth factors (Davies & Lindsay, 1985). Finally, we would expect age related changes to be

more extreme in neurons with long processes since sensory loss with aging is especially noticeable in distal extremities. Sensory threshold increases with age are most marked in the foot (Kenshalo, 1986; Mitchell & Schady, 1988). Thus any cell loss associated with these sensory losses might be expected to be much more noticeable in lumbar ganglia projecting to the foot than in the trigeminal ganglion. Further studies of SP-LI in lumbar DRGs from all ages are in progress to establish whether the percentage of SP-LI positive cells does increase with age in these lumbar ganglia.

The method of estimating the proportion of the cell area which contains lipofuscin is not ideal, as the pigment tends to accumulate at one pole of the cell (see Fig. 1), and the proportion seen therefore depends to some extent on the plane of the section. The effect of this will be to increase the variability of the data. However, a marked difference between neurones if it existed, should have become apparent with the large numbers of cell profiles evaluated in this study, but we found no evidence of any selective accumulation of lipofuscin in larger cells as has been reported to occur in rat DRGs with age (Van den Bosch de Aguilar & Vanneste, 1981). Indeed, if anything, the converse appears to be the case, in that a higher proportion of lipofuscin positive than of negative cell profiles were labelled by the small cell markers SP and SSEA4. However, in view of the studies on the aging rat (Van den Bosch de Aguilar & Vanneste, 1981), necessarily over a much shorter time course than these studies, there is a possibility that any initial differential accumulation may have been obscured or altered either by differential cell death or by the long term lipofuscin accumulation in these elderly subjects. Studies of tissue from a variety of ages are needed to clarify this point.

There was good consistency of size ranges of the distributions of positive and negative cell profiles between the three subjects, although there was less consistency between the percentages of labelled cells for each subject. This may indicate similar original neuronal populations in all ganglia with a superimposed variability in cell death according to the age, genetic background or medical history of the patient. Since in this study none of the subjects had suffered from a disease process known to cause sensory neuropathy, medical history is unlikely to have played a very large part in any differences seen.

With aging in the human there is a preferential deterioration of certain types of somatic sensation such as vibration and pressure sense especially in the lower extremities (Kenshalo, 1986; Mitchell & Schady, 1988). In contrast, many studies show no significant differences in thermal thresholds for cold or heat pain, although some others do report significant changes in thresholds particularly in the feet (Mitchell & Schady, 1988). These changes could result from a preferential loss of large diameter fibres. Furthermore, a decrease in myelinated fibre density occurs with aging in the sural nerve e.g. (Ro & Jacobs, 1993), while there is no agreement about a similar decrease in unmyelinated fibres (Jacobs & Love, 1985; Kanda *et al.*, 1991) perhaps because the decrease, if any, is less marked. A preferential loss of large myelinated fibres with a loss of low threshold mechanoreceptor information with age may result from the preferential loss of the larger, neurofilament-rich, somata in these elderly subjects, since there is a loose correlation between soma diameter and conduction velocity at least in rat and cat DRGs (Harper & Lawson, 1985; Lee *et al.*, 1986; Lawson & Waddell, 1991). This type of loss could account for the skew to the right of the neurofilament rich population. However, if this were the case, then ganglia from younger subjects should have higher proportions of neurofilament somata in their lumbar DRGs. Such a loss could also account for the elevated percentage of SP-LI positive neurons in these DRGs. However, another possible influence on the SP-LI containing neurons could be the availability of nerve growth factor from the periphery. If this was reduced (perhaps by fibre loss or damage) it might lead to down regulation of SP synthesis, perhaps even to cell loss. However there is not clear evidence for loss of the smaller nerve fibres in the sural nerve (see above). Furthermore, it is not yet known how important an influence growth factor availability is on the survival of aging sensory neurons in the human, although a reduction of NGF receptor mRNA in aging rat DRG neurons has been demonstrated (Buck *et al.*, 1987). Further studies are underway to examine how the proportions of human DRG neurons with SP-LI or with neurofilament rich cell bodies change with age.

Acknowledgements

We thank Barbara Carruthers and Perry Robbins for technical assistance, B. Anderton, P. Goodfellow and M. Portier for gifts of antibody and the MRC, UK and the Rushton Fund of the Physiological Society for financial support.

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