Histol Histopathol (2006) 21: 41-51

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Investigation of general and cytoskeletal markers to estimate numbers and proportions of neurons in the human intestine

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Summary. An important requirement in pathological diagnostics in the human enteric nervous system (ENS) is the estimation of the total numbers of neurons and of proportions of distinct subpopulations. In this study, we compared the suitability of two suggested panneuronal markers, cuprolinic blue (CB) and anti-Hu-protein (HU), for staining and counting human myenteric neurons in wholemounts, derived from small and large intestinal samples. Furthermore, the proportional expression of three cytoskeletal intermediate filaments, α-internexin (IN), neurofilament 200 (NF) and peripherin (PE), was correlated with both CB and HU. In 8 CB- and HU-stained wholemounts, 93.3% of all neurons were double labeled, 3.3% of neurons were stained only with CB whereas 3.3% were immuno-stained only for HU. Thus, both markers were comparably reliable in representing the putative total human myenteric neuron population in our material. The wholemounts double stained for IN/CB or IN/HU revealed between 56.2 and 71.5% of neurons to be IN-reactive. Between 42.8 and 50.9% of neurons were immunoreactive for NF whereas 53.9 to 62.4% of neurons were reactive for PE. Although our sample number was too small to allow final conclusions, we suggest that the variations in proportions of intermediate filament expression we observed may be due to individual circumstances rather than to correlation with age or region. The proportions of neurons positive for IN, NF or PE but unstained by CB histochemical or HU immunohistochemical techniques was between 0 and 2.2%. We conclude that both CB and HU techniques are suitable methods for representation of almost all myenteric neurons in the human gut and that the differential expression of the cytoskeletal proteins investigated has to be included in the classification of enteric neurons in pathological diagnostics of human gastrointestinal diseases.

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Key words: Enteric nervous system, Gut, Innervation, Intermediate filaments

Introduction

The enteric nervous system (ENS) is a complex neural network embedded in the wall of the gut (Furness and Costa, 1987). Studies in several species, most advanced in the guinea-pig, revealed a variety of different neuron types subserving specific functions in enteric circuits (Costa et al., 1996; Furness, 2000). A number of attempts were made to relate the proportions of these neuron types to the putative whole enteric neuron population. For this purpose, numerous putative general neuronal markers have been evaluated and their reliability for representation of all enteric neurons has been compared.

In the guinea-pig intestine, Karaosmanoglu et al. (1996) compared several candidate markers including cuprolinic blue (CB), neuron specific enolase (NSE), protein gene product (PGP) 9.5 and a Fos related antigen. The authors confirmed earlier results that NSE and PGP 9.5 stain most but not all enteric neurons and recommended cuprolinic blue as the method of choice for complete visualization of enteric neurons. CB binds selectively to the single-stranded RNA in neuronal nucleoli and Nissl substance and produces a stained cytoplasm combined with a prominent nucleolus against a pale blue or colourless background as has been demonstrated in various species (Heinicke et al., 1987; Holst and Powley, 1995; Román et al., 2001; Brehmer et al., 2002). Due to the relatively faint staining product of the CB technique, attempts were undertaken to enhance the intensity by application of microwaves and several consecutive incubation steps (Van Ginneken et al., 1998).

In the rat intestine, Phillips et al. (2004a) compared the efficacy of CB staining with HU and PGP 9.5 immunostaining as well as with intraperitoneal FluoroGold injections. They found that CB and HU were

comparably effective in representing the putative whole myenteric neuron population. HU-proteins occur solely in neurons and are an important factor for neuronal survival because they stabilize several messenger RNAs (DeGiorgio et al., 2003). HU selectively labels the neuronal nuclei and cytoplasm whereas the processes are not stained (Gultekin et al., 1998; Lin et al., 2002).

The neuronal intermediate filaments, neurofilaments (NF), peripherin (PE) and α-internexin (IN), are expressed in developing and/or adult mammalian neurons (Ho and Liem, 1996) and have also been found in enteric neurons (Eaker and Sallustio, 1994; Eaker, 1997).

The NF triplet proteins (L for low; M for medium; H for high molecular weight subunits) are class IV neuronal intermediate filaments (McGraw et al., 2002). NF are responsible for the organisation and maintenance of neuronal shape and function (Eaker, 1997; Julien, 1999; Ferrer-Alcon et al., 2000) and are present in subpopulations of myenteric neurons (Eaker, 1997).

IN (Pachter and Liem, 1985) is a class IV neuronal intermediate filament which has been described as a marker for immature, developing or regenerating neurons (Chien et al., 1998; Evans et al., 2002; McGraw et al., 2002). Faussone-Pellegrini et al. (1999) found the expression of IN to decrease with age in a myenteric neuronal lineage during mouse embryonic development. Immunoreactivity for IN has been demonstrated in enteric neurons of newborn but also adult rats (Eaker and Sallustio, 1994) as well as in those of older humans (Eaker, 1997).

PE, a type III intermediate filament, is upregulated in damaged, regenerating neurons (Terao et al., 2000) and was also suggested to be necessary for the development of subsets of neurons (Lariviere et al., 2002). This filament has been demonstrated in enteric neurons of rat and human, mostly in neurons immunonegative for NF (Eaker and Sallustio, 1994; Eaker, 1997).

We recently tried to define human myenteric neuron populations on the basis of their morphological and chemical phenotypes (Brehmer et al., 2004a,b, 2005). For the further development of neurohistopathological diagnostics it will be indispensable to relate the proportions of these neuron types to the whole enteric population. Thus, a panneuronal marker is necessary which should label the greatest number of neurons without simultaneous labeling of non-neuronal cells (Karaosmanoglu et al., 1996). Our first goal was to answer the question as to which of the two current, likely panneuronal markers, i.e. CB or HU, stain most of the myenteric neurons in human gut wholemounts. Since in our previous studies the shapes of neurons were represented by immunohistochemistry for NF, the second goal of this study was to assess the proportions of neurons which are immunoreactive for cytoskeletal proteins, including NF, IN and PE. This should provide a basis for our forthcoming studies which will estimate proportions of neuron types defined morphochemically in both healthy and pathologically altered human gut.

Materials and methods

Tissue processing and fixation

The use of human intestine for this study was approved by the ethics committee of the University of Erlangen-Nuremberg. The human tissue samples derived from 15 patients undergoing surgery for carcinoma. Only tissue from the margins of resected gut segments not affected by the tumour was used. Altogether 26 wholemounts from 15 patients were investigated (Table 1).

Gut segments were transported in physiological saline on ice (pH 7.3) as fast as possible to the laboratory. After arrival (approx. 1 to 5 hours after resection), they were rinsed in phosphate buffer at room temperature and transferred to Dulbecco's modified Eagle's medium (DME/F12-Ham; Sigma Chemicals, St. Louis, MO, USA), containing 10mg/ml antibiotic-antimycotic (Sigma), 50 µg/ml gentamycin (Sigma), 10% fetal bovine serum (Sigma), 4 µM nicardipine and 2.1mg/ml $NaHCO_3$, bubbled with 95% O_2 and 5% CO_2 at 37°C for 3 hours. After adding 100 µM colchicine, this bath was continued for a further 3 to 6 hours.

After this, samples were stretched and pinned on a Sylgard lined Petri dish, fixed in 4% paraformaldehyde, dissolved in 0.1 M phosphate buffer (pH 7.3) for 2 to 3 h at room temperature and subsequently washed several times in phosphate buffer.

Thereafter, 26 wholemounts (each about 2x1 cm) were prepared by scraping off the mucosa and removing the submucous and circular muscle layers using fine forceps. This resulted in longitudinal muscle/myenteric plexus wholemounts which were subjected to the following staining protocols.

Table 1. Patient and tissue characteristics.

AGE (years)	SEX	SEGMENT	TUMOR
18	Male	Ileum	Caecum carcinoid
21	Female	Transverse colon	Colon carcinoma
39	Female	Sigmoid colon	Colon carcinoma
42	Female	Duodenum	Pancreas carcinoma
54	Male	Ileum	Caecum carcinoma
54	Male	Sigmoid colon	Rectum carcinoma
60	Female	Duodenum	Pancreas carcinoma
60	Male	Duodenum	Pancreas cystadenoma
62	Female	Transverse colon	Colon carcinoma
70	Female	Ileum	Caecum carcinoma
72	Female	Sigmoid colon	Rectum carcinoma
73	Female	Sigmoid colon	Colon carcinoma
76	Female	Ileum	Caecum carcinoma
77	Male	Sigmoid colon	Colon carcinoma
80	Female	Sigmoid colon	Rectum carcinoma

Combined CB and single immunostaining

17 wholemounts were rinsed in a solution containing 0.025 M sodium acetate and 1 M $MgCl_2$ (pH 4.4-4.5). Afterwards, the gut wholemounts were incubated for 2 hours (42°C) in the same solution but with the addition of 0.3% CB (quinolinic phtalocyanine; Polysciences). Then, the samples were rinsed on a shaking table in distilled water (1 min), in sodium acetate plus 1 M $MgCl_2$ (3 times for 1 min) and in phosphate buffer (three times for 5 minutes).

The wholemounts were then preincubated for 2 hours in tris-buffered saline (TBS; pH 7.4) containing 1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.05% Thimerosal and 5% normal donkey serum on a shaking table. After rinsing for 10 min in TBS, the samples were incubated in TBS, 1% BSA, 0.5% Triton X-100, 0.05% Thimerosal and the primary antibodies (Table 2) for 3 days at 4°C on a shaking table following which they were washed out in TBS at 4°C for one day. Afterwards, the secondary antibodies (Table 2), dissolved in TBS, 1% BSA, 0.5% Triton X-100 and 0.05% Thimerosal (4 hours, room temperature, shaking table) were added. The wholemounts were rinsed again in TBS (1 day, 4°C, shaking table). We used wholemounts incubated in solutions lacking primary antisera which yielded no fluorescent signal as negative controls.

Double labeling immunohistochemistry

A further 9 wholemounts were preincubated for 2 hours at room temperature in the same solution as mentioned above for the immunostaining after which they were rinsed for 10 minutes in TBS on a shaking table. HU was used for all wholemounts, whereas the antibodies against NF, IN and PE were used for three preparations each. The incubations with primary and subsequently with secondary antibodies were carried out as described above.

Anti-lipofuscin treatment

After completing the above staining procedures, the wholemounts were transferred into a solution containing 1mM $CuSO_4$ in ammonium acetate buffer (pH 5.0) for 90 min followed by a short rinse in distilled water in order to reduce lipofuscin induced autofluorescence (Schnell et al., 1999; Brehmer et al., 2004a). Finally, the wholemounts were mounted in TBS-glycerol (1:1, pH 8.6).

Image processing

Neuron counting was performed on 260 ganglia, ten from each preparation. They were selected randomly in a meander-like fashion, provided that they contained at least ten CB and/or HU stained neurons to include as many neurons as possible. At present, there are no data derived from laboratory animals or human which indicate that ganglionic size correlates with neuron type composition, i.e., larger ganglia do not contain other neuron types than small ganglia.

Micrographs of 17 wholemounts counterstained with CB were collected using a digital camera system (Spot-RT-realtime, Visitron Systems, Munich, Germany) attached to a Leica Aristoplan microscope (40x dry objective) and SPOT advanced software (Version 3.5.6 for Windows, Diagnostic Instruments, USA). Images of the 9 HU counterstained preparations were created by using a confocal laser scanning microscope (BioRad MRC 1000, attached to a Nikon Diaphot 300, equipped

Table 2. List of antibodies used.

PRIMARY ANTISERA

Antigen	Host	Dilution	Source
Human neuronal protein HuC/HuD	Mouse	1:50	A-21271; Molecular Probes
Neurofilament 200	Mouse	1:200	N 0142; Sigma
Neurofilament 200	Rabbit	1:200	N 4142; Sigma
α-Internexin	Rabbit	1:1500	NB 300-139; Novus Biologicals
Peripherin	Goat	1:200	SC-7604; Santa Cruz Biotechnologies
Peripherin	Rabbit	1:100	AB 1530; Chemicon

SECONDARY ANTISERA

	Dilution	Source
ALEXA Fluor 488 Donkey anti-mouse IgG (H+L)	1:1000	A-21202; Molecular Probes
ALEXA Fluor 488 Donkey anti-rabbit IgG (H+L)	1:1000	A-21206; Molecular Probes
CyTM3-conjugated AffiniPure Donkey anti-mouse IgG (H+L)	1:800	715-165-151; Jackson Immunoresearch Labs.

with a krypton-argon laser; American Laser Corporation, Salt Lake City, UT, USA). Single optical section images through ganglia on the same focus plane were created by applying two different excitation wave lengths. The filter settings were 568 nm excitation/filter 605 DF322 (Cy3) and 488 nm excitation/filter 522 DF32 (Alexa). A 20x dry objective (numerical aperture 0.75) was used, the zoom factor was set to 1.5 in all scanning sessions.

Pairs of ganglion portraits were prepared using Confocal Assistant 4.02, Adobe Photoshop 6.0 and CorelDraw 11 (Figs. 1-7).

Quantitative evaluation of neurons

To enable counting of reactive neurons, a transparent plastic sheet was placed over the computer screen. Neurons were regarded as stained if nucleoli and/or perikarya (CB-staining), nuclei and/or perikarya (HU-staining) or perikarya (IN, NF, PE) exhibited signals. Labeled neurons in the pairs of images were marked with different colours for different stainings. All counts were performed by the same examiner.

Results

Qualitative observations

In general, longitudinal muscle/myenteric plexus wholemounts from different patients differed markedly in their amount of connective tissue between muscle layers and in the thickness of musculature. This resulted in differences in their staining intensities and qualities.

CB labeling revealed stained perikarya and nucleoli but a non-stained nucleus. In some relatively thin wholemounts the background staining was weak and the identification of the neurons was easy due to intense staining. In the cases of relatively thick specimens, the darker background rendered identification of neurons more difficult.

HU immunofluorescence, which labeled myenteric neurons brightly on a dark background, enabled the identification of most neurons, also in preparations containing a number of weakly colored neurons. The staining of reactive neurons was variable. In most cases, both the nucleus and the soma were strongly stained (Figs. 1b, 5a, 6a) whereas the nucleoli remained unstained. In some preparations, the soma showed strong but the nucleus only weak staining (Fig. 7a). In addition to this usual HU-staining pattern of neuronal nuclei and cytoplasm, two specimens also revealed labeling of intraganglionic nerve fibres (Figs. 5a, 7a).

Immunostaining for cytoskeletal filaments IN, NF and PE displayed both cell bodies and processes (Figs. 2b-7b). The intensity of staining, especially in the perikarya, varied from intense to weak. As could be observed in double labeling experiments, a number of perikarya were not stained for IN, NF or PE. In cases of weak reactivity, the numerous well stained intraganglionic nerve fibres may have obscured a potentially weak reactivity of some somata.

Quantitative evaluation

CB/HU stained wholemounts (Table 3)

The proportions of neurons simultaneously stained by both techniques was between 91.1% (sigmoid colon, 77 years) and 97.7% (duodenum, 42 years). No more than 4.7% of neurons (in the sigmoid colon of the 80 year old patient) were labelled with only one of the two markers used.

IN/CB and IN/HU stained wholemounts (Table 4)

Apart from the duodenal specimen from the patient aged 60 years (56.2%; see below), the proportions of IN-reactive neurons ranged between 67.9% and 71.5% (difference: 3.6%). The number of neurons positive for IN but negative for CB or HU, respectively, was negligible (between 0 an 0.8%). Comparing the 3 segments from the ileum, 2 derived from elderly patients (70 and 76 years old) and one from the 18 year old patient, it is obvious that the proportions of IN-positive neurons differ only slightly between the different age groups (between 69.1% and 71.5%). In contrast, the two duodenal specimens (42 and 60 years old) revealed a remarkable difference (69 versus 56.2%).

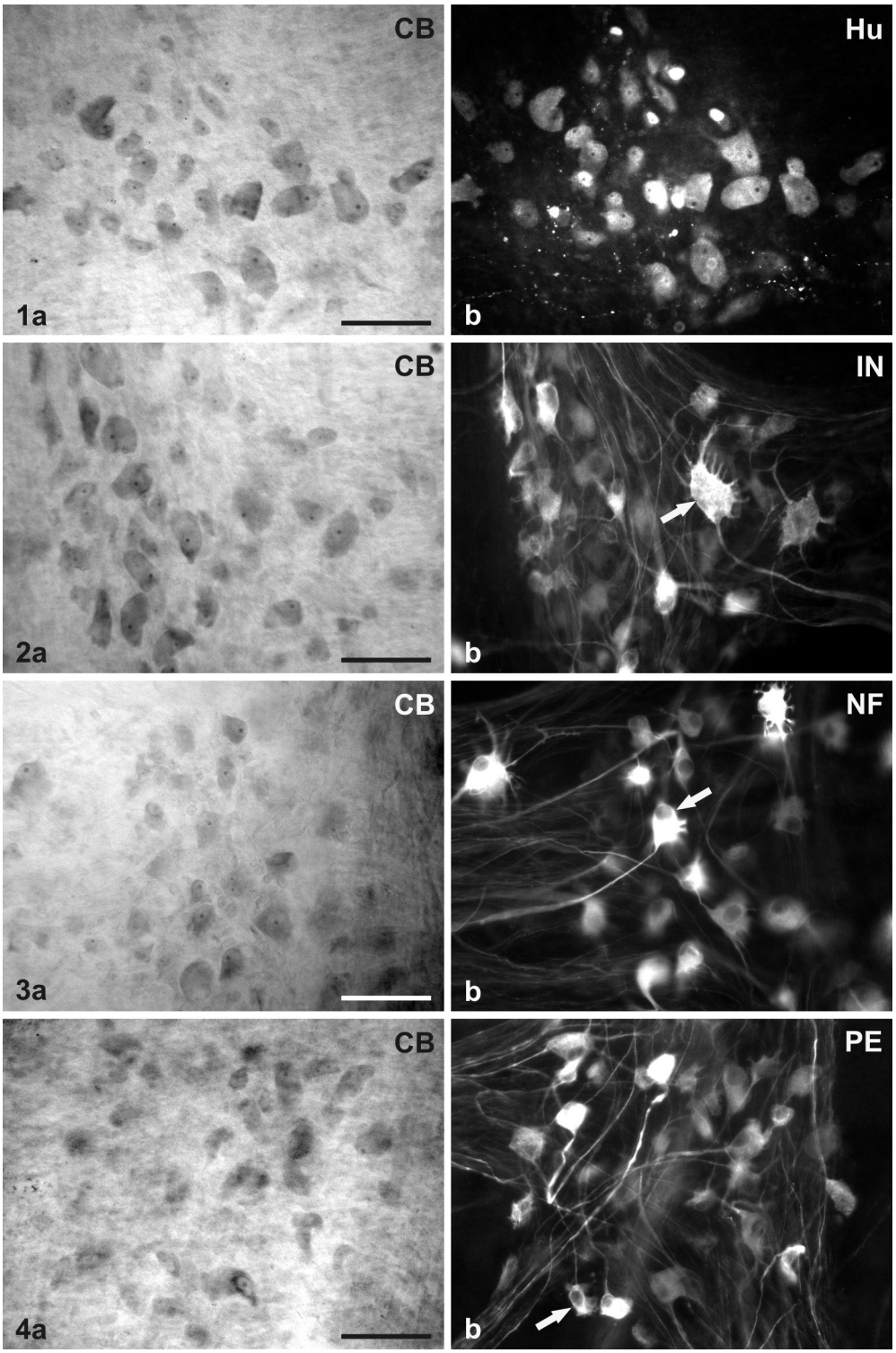
NF/CB and NF/HU stained wholemounts (Table 5)

The proportions of NF-stained neurons ranged between 42.8% and 50.9% (difference: 8.1%). Similar to

Table 3. Counts of neurons (and percentages) of 10 ganglia each in wholemounts (stained for HU and CB) derived from 8 patients.

AGE OF PATIENTS, SEGMENT	NEURONS (100%)	COSTAINED HU/CB	ONLY HU	ONLY CB
18, ileum	576	529 (91.8%)	22 (3.8%)	25 (4.3%)
39, sigmoid colon	583	540 (92.6%)	18 (3.1%)	25 (4.3%)
42, duodenum	614	600 (97.7%)	5 (0.8%)	9 (1.5%)
60, duodenum	573	528 (92.1%)	24 (4.2%)	21 (3.7%)
62, transverse colon	471	449 (95.3%)	12 (2.6%)	10 (2.1%)
70, ileum	454	421 (92.7%)	15 (3.3%)	18 (4.0%)
77, sigmoid colon	496	452 (91.1%)	22 (4.4%)	22 (4.4%)
80, sigmoid colon	570	528 (92.6%)	27 (4.7%)	15 (2.6%)
Σ	4337	4047 (93.3%)	145 (3.3%)	145 (3.3%)

the IN-stained specimens, the number of neurons positive for NF but negative for CB or HU was negligible (between 0 an 0.3%). Two segments from sigmoid colon of comparable age displayed a greater difference in the proportions of NF-positive neurons (48.2 versus 42.8%) whereas proportions of different



Figs. 1-4. Myenteric ganglia stained with cuprolinic blue (CB; **1a-4a**) technique and, subsequently, co-immunostained for anti-Hu-protein (HU; **1b**; specimen from a patient aged 39 years, sigmoid colon), α-internexin (IN; **2b**; 42 years old, duodenum), neurofilament 200 (NF; **3b**; 54 years old, sigmoid colon) and peripherin (PE; **4b**; 70 years old, ileum). CB-staining **(1a-4a)** revealed nucleoli as well as neuronal cytoplasm to be marked with variable intensity, neuronal processes were not stained. Hu-staining **(1b)** resulted in brightly stained nuclei and cytoplasm whereas nucleoli appear as dark (unstained) points, processes were not stained. Staining with the intermediate filaments, IN, NF and PE **(2b-4b)**, revealed both cytoplasm and processes to be stained. In some neurons, axon and dendrites could be distinguished (arrows). Bars: 50 µm.

segments (ileum, 76 year old versus sigmoid colon, 73 year old) differ less markedly (50.9 versus 48.2%).

PE/CB and PE/HU stained wholemounts (Table 6)

Similar to the proportions for IN in Table 4, the duodenal specimen from the 60 year old patient also displayed the lowest proportions in PE-stained wholemounts, 53.9%. Apart from this, PE-positive neurons ranged between 57.8% and 62.4% (difference: 4.6%). The number of neurons positive for PE but negative for CB or HU was between 0 and 2.2%. In the 2 ileal specimens stained for PE, the values differed only slightly (60.6 versus 62.4%) despite the marked difference in age of the patients (18 versus 76 years).

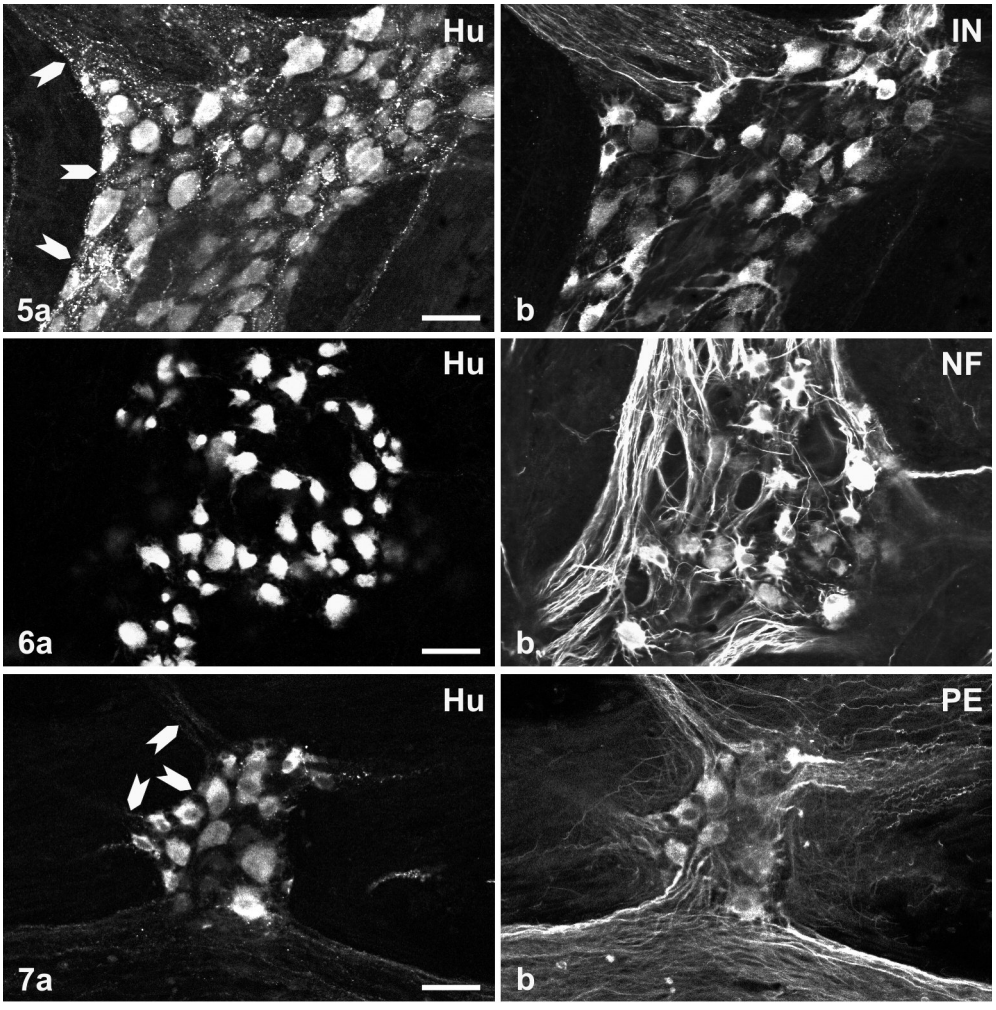
The duodenal specimens from the 60 year old patient display by far the lowest proportions in both the IN- and PE-stained groups as is obvious from Tables 4 and 6.

Discussion

General remarks

In this study, we counterstained human myenteric plexus wholemounts derived from different gut segments from patients of different ages with CB and HU, two suggested panneuronal markers in order to estimate which of the two methods represents the maximum number of myenteric neurons. Based on counts of neurons of ten randomly selected ganglia in each wholemount, visualized by one or both of these techniques, we conclude that they are comparably reliable in representing the entire human myenteric neuron population (see below). Until there is another method available which demonstrates more neurons, we consider them to be panneuronal markers.

Furthermore, we estimated the proportions of



Figs. 5-7. Myenteric ganglia double-immunostained for anti-Hu-protein (HU) and α-internexin (IN; **5a,b**; specimen from a patient aged 18 years old, ileum), HU and neurofilament 200 (NF; **6a,b**; 54 years old, ileum) as well as HU and peripherin (PE; **7a,b**; 77 years old, sigmoid colon). In addition to the usual nuclear and cytoplasmic HU-staining of neuronal perikarya, 2 specimens also displayed labeling of nerve fibres (more strongly in Fig. 5a, less strongly in Fig. **7a**) thus outlining the putative ganglionic boundaries (examples are marked with arrowheads). Bars: 50 µm.

neurons immunoreactive for cytoskeletal markers including IN, NF and PE as related to neurons stained for CB or HU, i.e., the putative whole myenteric population. We were forced to present data derived from patients of different ages and with different pathological conditions and from different gut segments with different transit times between resection and arrival at the laboratory. Consequently, we presented the results of single counts, thus supplying value ranges for comparison with future data. We desisted from further statistical treatment of data (mean values, standard deviations) since, in our opinion, this would have resulted in overinterpretation of the findings. It is well known, from data derived both from laboratory animals and humans, that numbers and proportions of neurons vary according to gastrointestinal region, age and pathological circumstance (Schuffler et al., 1978; Schuffler and Jonak, 1982; Krishnamurthy, 1985;

Table 4. Counts (and percentages) of neurons of 10 ganglia each in 3 wholemounts stained for IN and CB (upper part) and in 3 wholemounts stained for IN and HU (lower part).

AGE OF PATIENTS, SEGMENT	NEURONS (100%)	COSTAINED IN/CB	ONLY IN	ONLY CB
42, duodenum	603	416 (69.0%)	0 (0.0%)	187 (31.0%)
60, duodenum	523	294 (56.2%)	4 (0.8%)	225 (43.0%)
70, ileum	472	326 (69.1%)	0 (0.0%)	146 (30.9%)
Σ	1598	1036 (64.8%)	4 (0.3%)	558 (34.9%)
		COSTAINED IN/HU	ONLY IN	ONLY HU
18, ileum	421	301 (71.5%)	0 (0%)	120 (28.5%)
54, sigmoid colon	445	302 (67.9%)	0 (0%)	143 (32.1%)
76, ileum	641	456 (71.1%)	2 (0.3%)	183 (28.6%)
Σ	1507	1059 (70.3%)	2 (0.1%)	446 (29.6%)

Table 5. Counts (and percentages) of neurons of 10 ganglia each in 3 wholemounts stained for NF and CB (upper part) and in 3 wholemounts stained for NF and HU (lower part).

AGE OF PATIENTS, SEGMENT	NEURONS (100%)	COSTAINED NF/CB	ONLY NF	ONLY CB
54, sigmoid colon	606	270 (44.6%)	0 (0.0%)	336 (55.4%)
73, sigmoid colon	610	294 (48.2%)	1 (0.2%)	315 (51.6%)
76, ileum	550	280 (50.9%)	0 (0.0%)	270 (49.1%)
Σ	1766	844 (47.8%)	1 (0.1%)	921 (52.2%)
		COSTAINED NF/HU	ONLY NF	ONLY HU
54, ileum	443	193 (43.6%)	1 (0.2%)	249 (56.2%)
62, transverse colon	394	173 (43.9%)	1 (0.3%)	220 (55.8%)
72, sigmoid colon	516	221 (42.8%)	1 (0.2%)	294 (57.0%)
Σ	1353	587 (43.4%)	3 (0.2%)	763 (56.4%)

Table 6. Counts (and percentages) of neurons of 10 ganglia each in 3 wholemounts stained for PE and CB (upper part) and in 3 wholemounts stained for PE and HU (lower part).

AGE OF PATIENTS, SEGMENT	NEURONS (100%)	COSTAINED PE/CB	ONLY PE	ONLY CB
21, transverse colon	472	293 (62.1%)	0 (0.0%)	179 (37.9%)
42, duodenum	566	327 (57.8%)	3 (0.5%)	236 (41.7%)
76, ileum	554	336 (60.6%)	2 (0.4%)	216 (39.0%)
Σ	1592	956 (60.1%)	5 (0.3%)	631 (39.6%)
		COSTAINED PE/HU	ONLY PE	ONLY HU
18, ileum	314	196 (62.4%)	1 (0.3%)	117 (37.3%)
21, transverse colon	290	172 (59.3%)	3 (1.0%)	115 (39.7%)
60, duodenum	271	146 (53.9%)	6 (2.2%)	119 (43.9%)
Σ	875	514 (58.7%)	10 (1.1%)	351 (40.1%)

Cortesini et al., 1995; Belai et al., 1997; Belai and Burnstock, 1999; Jarvinen et al., 1999; Wester et al., 1999; Neunlist et al., 2003; Hanani et al., 2004; Román et al., 2004). Although it would be desirable to obtain standard data of a given gut segment derived from large numbers of age and sex matched patients with comparable pathological background, this is, however, almost impossible under realistic conditions, even if several surgical departments were involved as specimen providers. Although we found some variation in proportion of intermediate filament expression, it was not too marked. We suggest that it may be due to individual circumstances rather than to a correlation with age or region.

Panneuronal markers

A panneuronal marker should represent the maximum number of neurons without simultaneous labeling of non-neuronal cells (Karaosmanoglu et al., 1996). Both markers applied in this study on human tissues, CB and HU, were previously proposed to be suitable for accurate counting of the whole enteric neuronal population in laboratory animals. Phillips et al. (2004a) compared both protocols in the rat intestine and suggested them to be suitable for representation of the whole enteric neuron population.

Unfortunately, both methods also stain cells which are not or are perhaps not neurons. Heinicke et al. (1987) found the granules of mast cells were stained by the CB technique but, unlike blue colored neurons, they appeared purple, due to metachromasia. Pale blue-stained connective tissue cells (mainly fibroblasts) were found in the submucosa Holst and Powley (1995) and close to the smooth musculature surrounding the myenteric plexus (Van Ginneken et al., 1998). However, both elements were easy to differentiate from neurons. As to HU immunohistochemistry, Phillips et al. (2004a) observed very few HU-positive elements within myenteric ganglia coreactive with S-100, a marker suggested to be specific for glial elements. Nevertheless, they concluded that the specificity of HU immunostaining is sufficient for most purposes.

The qualitative differences between stainings both in CB and HU procedures were the main problem encountered in our material. In the case of HU, the age of the subjects from whom tissue was obtained may offer explanation. This has been found in the rat and also discussed by Phillips et al. (2004b). These authors suggested that the intensity of HU immunostaining may weaken due to downregulation of the Hu protein expression. Since most of our gut segments originated from elderly patients, the same may apply. We found few neurons negative for HU or CB but simultaneously positive for the other panneuronal marker (Table 3) or one of the intermediate filament-antibody used (Tables 4-6). We suggest that these neurons are not a special population with unique histochemical or immunohistochemical characteristics but rather that particular circumstances may be responsible for the negative staining results (penetration of agents, time period between resection and fixation with decrease of, e.g., antigen concentration etc.).

In two HU-stained specimens, we found that nerve fibres were also stained, in addition to stained neuronal somata. It is possible that upregulation of the antigen and its subsequent presence within some axons occurred either due to the age of the patient or the pathological background or unknown influences on the tissues between resection and fixation.

The intensity of CB also varied significantly. In some wholemounts, numerous neurons were very hard to identify. Phillips et al. (2004a) mentioned that, in the rat intestine, penetration of CB during the staining process reached the same level in cases of complete muscular wholemounts as compared with longitudinal muscle/myenteric wholemounts lacking the circular muscle layer. In our human wholemounts, the thicker the specimen (and the harder the dissection process) the less optimal the signal-to-noise-ratio was between the specific staining of the perikaryon and the background. The practical problem resulting from this may be that examiners with different experience may count different numbers of neurons. This subjectivity cannot be fully excluded and is a general problem throughout histochemistry and immunohistochemistry. Since all counts were done by one and the same examiner, the same criteria applied throughout our study.

Although we have tried to enhance the staining intensity of CB technique by applying microwave treatment during incubation (Van Ginneken et al., 1998), this was not unequivocally positive in our experience (unpublished observations). The intensity of a considerable number of neurons, even the less intensely stained ones, could not be increased. Furthermore, the morphological representation of neurons using, e.g., subsequent NF immunohistochemistry was suboptimal. Since the latter technique is necessary for our morpho-chemical classification experiments, we have abandoned this methodological modification.

Another contributing fact for the differing quality of specimens and stainings is the impossibility of applying standard conditions to human tissues. The human gut segments available (frequently only a few square cm in size) originate from different gut regions and from patients of different ages with different diseases. Also the time period between resection of the segment and its arrival in the laboratory ranges between one and several hours. Since our tissue specimens were provided by different hospitals, it was impossible to ensure an immediately subsequent time point (after resection) and an equivalent method of fixation for the different gut segments (e.g., degree of stretching during fixation). One of our reasons for applying organ bath procedures in culture medium for several hours after arrival of tissue samples in the laboratory was to ensure comparable conditions for all samples at least for the time point of fixation. It is possible that some neurons may have been

damaged and/or died during transit (between resection and arrival in the laboratory) or during the organ bath. It has been noted by Wood (1994) that electrophysiological recordings of neurons, stored in vitro at 5°C for 48 hours and rewarmed to 37°C, indicate that at least some enteric neurons are resistant to unphysiological conditions such as low temperature and hypoxia. Another reason for applying an organ bath was that especially the results of NF-staining after immediate fixation of tissues remained weak and incomplete (unpublished observation). Thus, we followed a protocol of Llewellyn-Smith et al. (1985), who suggested the organ bath prior to fixation, originally proposed for better preservation of ultrastructure. The reason for adding colchicine for the last hours of the organ bath is to ensure the same tissue conditions prior to immunohistochemistry which have been used in earlier (Brehmer et al., 2004a,b, 2005) and will be used in future studies. The main purpose of these studies was and will be the deciphering of both the morphological and chemical code of human enteric neurons. It is necessary to enhance the concentration of neuroactive peptides within cell bodies through colchicine pretreatment to enable their immunohistochemical detection. Standard numbers of enteric neurons and standard proportions of neuron types per gut segment age etc. are thus much harder to evaluate in humans compared to laboratory animals.

Nevertheless, it seems that in our specimens both CB and HU protocols revealed nearly the whole neuron population. The numbers of neurons labeled by only one of the two methods was smaller than 5% in all cases. The ranges observed (costaining of HU and CB in 91.1 to 97.7% of neurons) can be interpreted as interindividual variability. It seems unlikely that regional or age dependent variability exists concerning the affinity of neurons to the general neuronal markers applied.

Cytoskeletal markers

Our second aim was to estimate the proportions of neurons immunoreactive for three cytoskeletal proteins, i.e. IN, NF and PE. All three markers were counterstained with CB or HU. The only previous report dealing with co-immunoreactivity of human enteric neurons for IN, NF and PE was that of Eaker (1997). This author presented a pair of figures with costained neurons for NF and IN and estimated, without giving original data, the following proportions: "Using double-labeling with neurofilament and peripherin or α-internexin, we found 70% of immunoreactive neurons contained neurofilaments, 65% contained peripherin (nearly equivalent to staining with α-internexin), and 35% contained both neurofilament and peripherin." (Eaker, 1997). Since the author did not use any panneuronal marker, these proportions are difficult to compare with those of the present study. For adult rat myenteric neurons, Eaker and Sallustio (1994) presented more detailed results. In relation to the number of PGP9.5-positive and -negative neurons, 56% were IN-positive (between 56.2 and 71.5% in our human material), 65% were NF-positive (42.8 - 50.9%) and 53% (53.9-62.4%) were PE-positive. Our values from human intestine are within comparable ranges. We also noted another similarity between the results of Eaker and Sallustio (1994) in the rat and our results in humans. Numerous small neurons were preferentially positive for IN or PE, rather than for NF. Thus, the differential expression of neuronal intermediate filaments may be related to other morphological and/or chemical features of neuron populations and may indicate that these neurons belong to different subtypes.

Apart from the values obtained from the duodenal samples of the 60 year old patient, the variations in proportions of IN-, NF- and PE-stained neurons found in our material were not remarkable (IN: 3.6%, NF: 8.1%, PE: 4.6%). Taking into account the significant age differences (18-80 years) and the broad range of segments investigated (duodenum-sigmoid colon), the variations may rather be due to individual variability than to influence of age or region. Of course, this has to be addressed in future studies on a broader sampling basis.

Since the various components of the neuronal cytoskeleton may be involved in different pathological conditions (Brandt, 2001; Julien and Mushynski, 1998) the different neuron types that express these components may be afflicted by different diseases. Thus, we will include IN and PE in our future attempts to classify human enteric neurons and to decipher their plasticity in disease states.

Acknowledgements. We are grateful to Stephanie Link, Karin Löschner and Hedwig Symowski for their excellent technical assistance and to Patricia Heron for checking the manuscript. Furthermore, we thank Gerhard Seitz and Barbara Blaser (Bamberg), Thomas Papadopoulos, Arno Dimmler, Roland Croner, Bertram Reingruber and Martin Rexer (Erlangen) as well as Holger Rupprecht and Daniel Ditterich (Fürth) for their kind cooperation. This study has been supported by the Deutsche Forschungsgemeinschaft (BR 1815/3).

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Accepted September 5, 2005