Histol Histopathol (1996) 11: 769-786

Histology and Histopathology

Invited Review

Identification of the interstitial cells of Cajal

T. Komuro[1], K. Tokui [2] and D.S. Zhou[1]

[1]Department of Basic Human Sciences, Waseda University School of Human Sciences, Mikajima, Tokorozawa, Saitama and [2]Matsuyama Bethel Hospital, Matsuyama, Ehime, Japan

Summary. Observation of whole-mount stretch preparations using the zinc-iodide-osmic acid method reveals a wide variety of interstitial cells in different tissue layers of the guinea-pig small intestine. And a subsequent electron microscopic examination and survey of references makes clear that the interstitial cells of Cajal (ICC) depicted in original drawings of Cajal are heterogeneous and correspond to different types of interstitial cells. The myenteric ICC are characterized by long dichotomous branching processes which constitute cellular networks independent from the nerve plexus and form many gap junctions at their tips. Their ultrastructure is similar to that of fibroblasts and they have no basal lamina. The myenteric ICC show strong immunoreactivity for vimentin and the *c-kit* receptor, and probably correspond to the intestinal pacemaker cells.

Within the circular muscle layer, ICC are represented by the cells that are closely associated with fine nerve bundles. The ICC have various shapes, ranging from bipolar to stellate, depending on the running pattern of the nerve fibers that they are associated with. They show fibroblast-like ultrastructure and have no basal lamina. They form gap junctions with smooth muscle cells and are immunoreactive for vimentin.

On the other hand, ICC associated with the deep muscular plexus described in the guinea-pig by Cajal could not be clearly identified. However, it is suggested that the ICC in this location may correspond to glycogen-rich cells possessing a basal lamina. Although they show a fairly well-developed rough endoplasmic reticulum, Golgi apparatus and immunoreactivity for vimentin, ICC of the deep muscular plexus are probably specialized smooth muscle cells in nature.

Key words: Ultrastructure, *c-kit*, Vimentin, Intestine, Pacemaker

Offprint requests to: Professor Terumasa Komuro, Department of Basic Human Sciences, Waseda University School of Human Sciences, Mikajima 2-579-15, Tokorozawa, Saitama 359, Japan

Introduction

The great neuroanatomist, Santiago Ramon y Cajal described «cellules interstitielles (or neurones sympathiques interstitiels)» in association with the terminal arborization of the autonomic nerves of intestines, glands and blood vessels stained with methylene blue or the Golgi method (Cajal, 1893, 1911). Ever since then, interstitial cells of Cajal (ICC), as referred to by following microscopists, have been a subject of a historical debate with respect to their cytological nature. The cells located in the interstitium of the intestinal wall, in particular, have received special attention by many investigators (see reviews by Boeke, 1949; Meyling, 1953; Taxi, 1965).

Among others, Taxi (1965) maintained the opinion that ICC were neural in nature and were distinct from fibroblasts, whereas Richardson (1958, 1960) was sceptical about the presence of two distinct cell types and suggested that ICC were fibroblasts, based on observations of silver impregnation of the rabbit small intestine.

Modern ICC research was revived by Thuneberg (1982) who proposed the novel hypothesis that ICC act as pacemakers and have an impulse conductive function in the intestinal musculature analogous to those of the heart muscle, as suggested earlier by Keith (1914/15, 1915, 1916). Indeed, recent physiological studies have accumulated evidence that ICC are involved in the generation of the slow waves which represent the electrical signals of the pacemaker function (Hara et al., 1986; Suzuki et al., 1986; Huizinga et al., 1988; Du and Conklin, 1989; Conklin and Du, 1990; Serio et al., 1990; Liu et al., 1993, 1994). Consequently, these cells have become central for an understanding of intestinal movement.

However, the cytological definition and the developmental origin of ICC remain unsettled. Part of the confusion seems to result from a variety of cells observed in different tissue layers in different portions of the alimentary tract, in different species (see reviews by Thuneberg, 1989; Christensen, 1992). Another important reason for the uncertainty is the lack of a truly specific

staining method for ICC. In view of these problems, Thuneberg (1982, 1989) advanced the understanding of ICC by making an extensive survey of the literature, and by the classification of these cells on the basis of their location and ultrastructure. Christensen (1992) raised several significant questions about ICC, attempting to answer them by making a broad survey of the literature, but was able to establish only rather vague cytological criteria for ICC.

More recently, several new approaches to identifying ICC by (immuno)histochemical methods including NADH diaphorase histochemistry (Xue et al., 1993), Chorela toxin subunit b labelling (Anderson and Edwards, 1993), cyclic GMP immunoreactivity (Shuttleworth et al., 1993; Young et al., 1993) and NO synthase immunoreactivity (Xue et al., 1994) have been developed. However, again, none of them have been considered as being truly specific for ICC.

When ICC research enters a new phase, such as to discuss a certain gene (*c-kit*) in association with intestinal pacemaker activity (Ward et al., 1994; Huizinga et al., 1995), it is essential to establish a set of clear-cut criteria for ICC to support the further development of ICC research. For instance, studies of cytodifferentiation of ICC (Faussone-Pellegrini, 1984, 1985, 1987) must be understood for establishing the morphological criteria of matured specimens.

To exclude ambiguity from a definition of ICC, it is important to identify them on the basis of a good correlation between a given cell type and those originally described by Cajal. Therefore, first of all, the present study elucidates the whole cell shape of a variety of interstitial cells located in different tissue layers in the guinea-pig small intestine by using whole-mount stretch preparations stained by a modified zinc iodide-osmic acid (ZIO) method (Rumessen and Thuneberg, 1982).

The ZIO method is not a specific staining for ICC: rather it stains nerve fibers and a certain range of interstitial cells which are likely to include cells with the features of ICC. This property of the ZIO method is shared with methylene blue and the Golgi method, which were originally used to observe ICC (Cajal, 1893, 1911). It is therefore a useful tool for identifying ICC. Good penetration ability is another advantage of the ZIO method, which makes it possible to observe any tissue layer of the whole-mount preparations of guinea-pig small intestine.

Immunohistochemistry for vimentin and the c-*kit* can also be applied to speculate about the developmental origin and functional role of ICC. Vimentin immunostaining has been used as a useful tool to observe ICC (Komuro, 1987; Komuro et al., 1994).

Further, we clarified the ultrastructural features of candidate cells by means of a critical correlation of their whole cell shapes with the original drawings of ICC (Cajal, 1893, 1911). This approach makes it possible to define clear-cut morphological criteria for ICC via integrating observations obtained by traditional histological staining and by modern methods.

Regarding nomenclature, the term ICC will be used only for those cells whose equivalence to the original description has been confirmed, or those which can be regarded as species variations of ICC. The rest of the cells, including those which have been regarded as ICC in the literature, without firm evidence, and the cells of the interstitium in a general sense, will be described simply as «interstitial cells» to avoid confusion.

In the following text, ICC at different locations will be described according to the terminology of Thuneberg et al. (1995) i.e., ICC-AP (Auerbach plexus) located between the circular and longitudinal muscle layers; ICC-DMP (deep muscular plexus) located between the inner thin and outer thick sublayers of the circular smooth muscle of the small intestine; ICC-SMP (submuscular plexus) located at the submucosal border of the colonic circular muscle layer; ICC-CM located within the outer thick circular muscle layer; and ICC-LM located within the longitudinal muscle layer. Cells in the subserous tissue layer are described simply as interstitial cells for the reason described above.

This article addresses only cytological identification of ICC and readers are advised to consult comprehensive review articles by Thuneberg (1982, 1989), Christensen (1992) and Thuneberg et al. (1995) for other aspects of ICC. A part of this study has been published elsewhere (Komuro et al., 1994).

Materials and methods

Zinc iodide-osmic acid (ZIO) staining

Short segments of guinea-pig small intestine were placed in a Tyrode solution containing 1mM papaverine to make a complete relaxation of the muscle cells for about 10 min, at an initial temperature of 37 °C (Rumessen and Thuneberg, 1982). Then, they were moderately inflated and fixed for 24 h at room temperature with fixative containing 0.4% OsO_4 and 2.4% ZnI_2. The specimens were then rinsed in distilled water and cut along the mesentery to make flat sheets. Under a dissecting microscope, circular muscle layers, longitudinal muscle layers, with or without the myenteric plexus, and serosa without serous epithelium were carefully dissected from remaining parts of the intestinal wall with fine forceps. These specimens were mounted with aqueous mounting medium Mount Quick (Daido Sangyo). For transmission electron microscopy, suitable areas of the specimens were cut out, block-stained with a saturated aqueous uranyl solution for 2 h and then processed for electron microscopy as described below.

Immunohistochemistry

Short segments of guinea-pig small intestine were inflated and fixed for either 2 h at 4 °C with fixative containing 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for vimentin and S-100, or 30 min at

4 °C with 100% acetone for c-*kit* receptor. After rinsing in phosphate-buffered saline (PBS) each layer of specimen was dissected as described above. The isolated pieces were placed in PBS containing 0.3% Triton X-100 at 4 °C for 5-10 min. The specimens were then stretched on a glass slide and were first incubated with 4% Block Ace solution (Dainippon Seiyaku) for 30 min at room temperature to avoid non-specific staining. The following steps for immunoreaction were separately performed for different antisera as follows: **vimentin-** Specimens were incubated overnight with the monoclonal antibody against vimentin (DAKO, V9; No. M725) at a dilution of 1:20. After washing in PBS several times, the specimens were incubated further overnight with peroxidase-conjugated secondary antibodies (DAKO, rabbit anti-mouse IgG) at a dilution of 1:100. Horseradish peroxidase reaction was developed in a solution of 6mg of 4-chloro-1-naphtol (CN; Sigma) in 50 ml 0.1M Tris-HC1 buffer (pH 7.4) with 8 µl of 30% H_2O_2. **c-kit receptor-** The isolated muscle layers were incubated overnight with the monoclonal antibody against the c-kit receptor (ACK-2; GIBCO, No 3314SA) at a dilution of 1:200. The peroxidase-conjugated secondary antibodies (goat anti-rat IgG, GIBCO 13860-010) were used at a dilution of 1:80 and the peroxidase reaction was performed with the same procedure as described above. **S-100-** Specimens were incubated overnight with anti-S-100 antibody (DAKO, Z311, rabbit polyclonal) at a dilution of 1:200. The primary antibody was visualized with biotin-streptavidin method by using BAS-PO kit (Biogenex Lab, San Ramon).

Cholinesterase reaction

Counterstaining of the myenteric plexus in the immunohistochemical preparations was performed by thiocholine method (Karnovsky and Roots, 1964) after the horseradish peroxidase reaction. In brief, the specimens were incubated for about 30 min at 37 °C in medium containing 5mg acetylthiocholine iodide, 0.5 ml 0.1M sodium citrate, 1ml 30mM $CuSO_4$, 1 ml distilled water and 1ml 5mM potassium ferricyanide in 6.5 ml 0.1M acetate buffer, pH 6.0.

Bauer's staining for glycogen

Short segments of guinea-pig small intestine were moderately inflated and fixed for 4 h with Bouin's fixative. After rinsing in PBS, the circular muscle layer was dissected, as described above. The specimens were stained by Bauer's glycogen staining (using Chromic acid instead of Periodic acid in the PAS reaction) to avoid coloration of the basal lamina of the smooth muscle cells around the target interstitial cells.

Transmission electron microscopy

Pieces of guinea-pig small intestine were placed in a fixative containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 2 h at 4 °C. The specimens were then rinsed in the same buffer and post-fixed in 1% OsO_4 in the same buffer for 2 h at 4 °C. Following osmication, the specimens were rinsed in distilled water, block-stained with a saturated aqueous uranyl acetate solution for 2 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon Epoxy resin. Ultrathin sections were cut using a Reichert ultramicrotome and double-stained with uranyl acetate and lead tartrate for observation with a JEOL JEM 1200EX II electron microscope.

Results

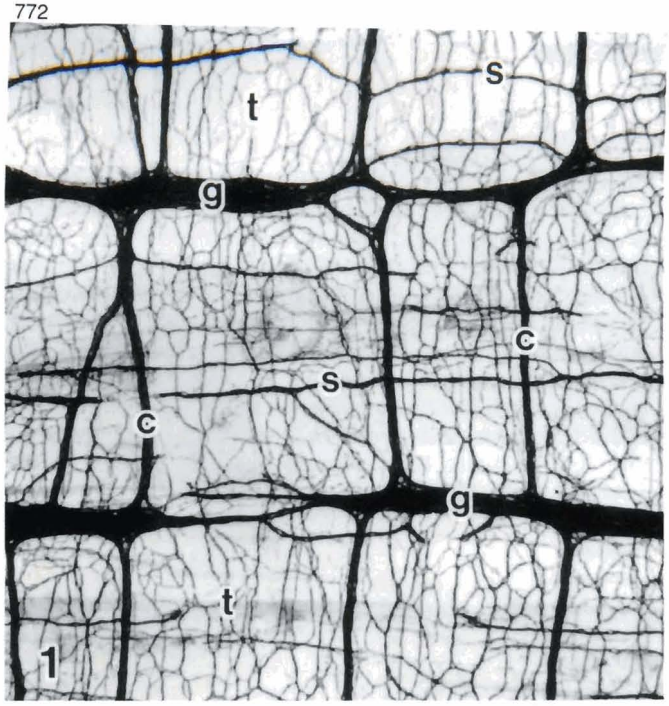
Cell shapes revealed by whole-mount stretch preparations

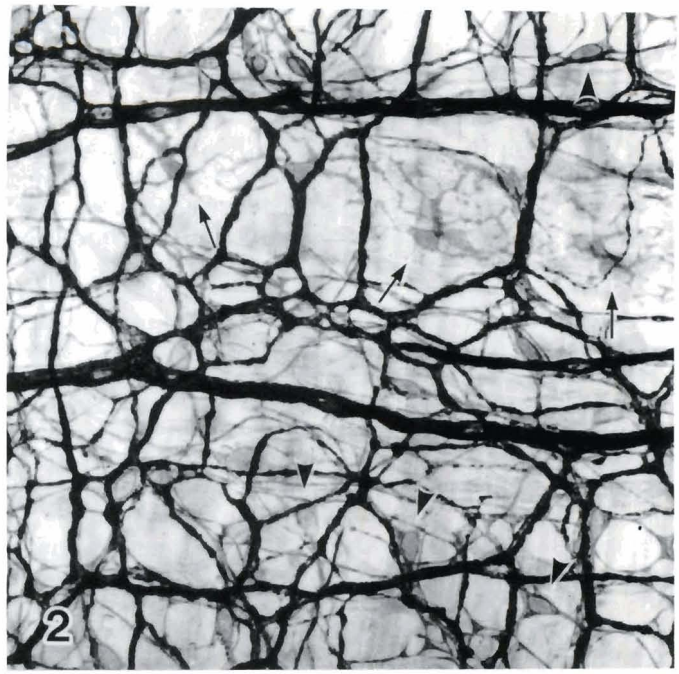
Region of the myenteric plexus

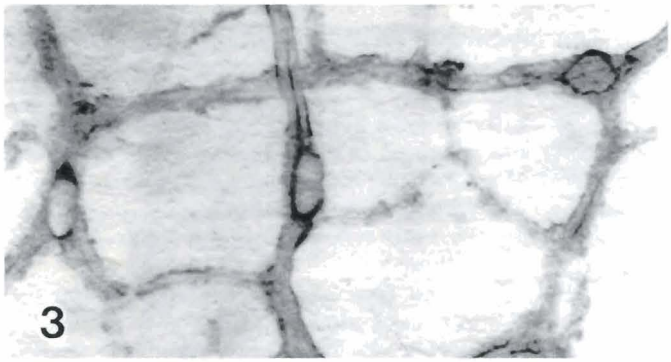
The myenteric plexus of the guinea-pig small intestine consists of three components: the primary, secondary and tertiary plexus (Fig. 1). Interstitial cells were generally stained using longer incubation times with ZIO and appeared in the interstices of the tertiary meshwork (Fig. 2). They were easily distinguished from Schwann cells which were located in the midst of nerve bundles (Fig. 3). Four types of cells were recognized using the ZIO method.

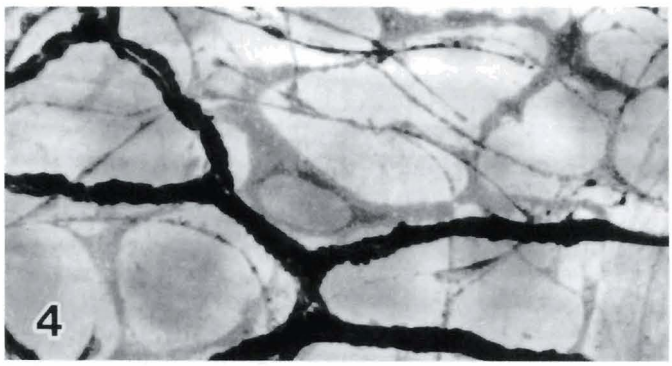
Cells of the first type were well stained using the ZIO method and usually showed darker gray cytoplasm with paler nuclei (Fig. 4). They were irregularly shaped cells with fairly large perinuclear cytoplasm and a few, broad primary processes with sharp, wavy contours. The nuclei were elongated with a longer diameter of about 12-18 µm and a shorter diameter of about 6-10 µm. In the cytoplasm, vacuoles and fat droplets were frequently seen in counter contrast. They had a close association with nerve bundles of the tertiary plexus. Therefore, the processes did not have a regular branching pattern, and were usually confined with adjacent meshes of the tertiary plexus. However, they occasionally sent out the processes perpendicular to the plane of the myenteric plexus and penetrated into the circular muscle layer, giving off further branches running parallel to the muscle cells. Although the cells made contact with processes of the same type of cells, they did not seem to constitute their own complete cellular network. Cells of this type were weakly stained with vimentin immunohistochemistry (Fig. 8).

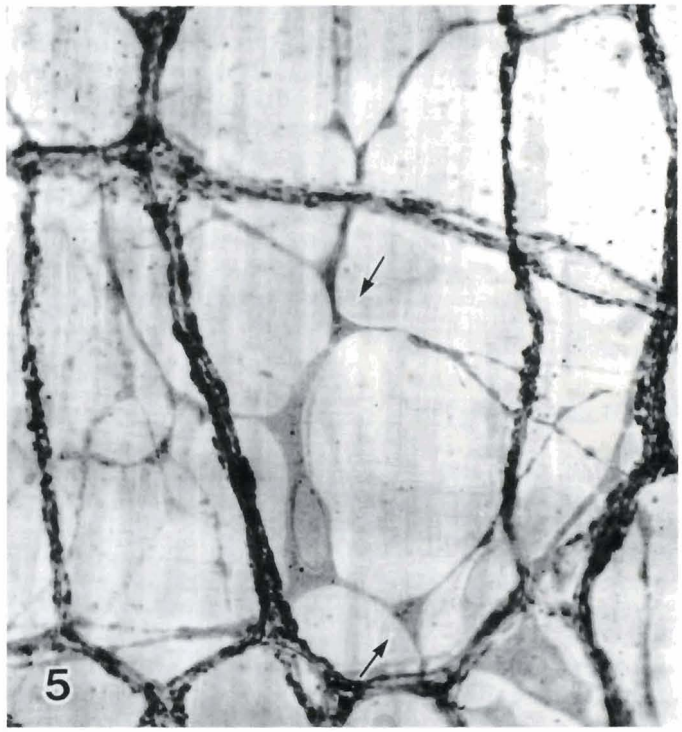
Cells of the second type were triangular or stellate cells with long slender processes with smooth and rather straight contours (Fig. 5). They were prominent and constituted the main population of interstitial cells in this region. They were often stained so weakly by ZIO that the cytoplasmic particulate could be seen through light gray cytoplasm. The nuclei were generally elongated and slightly larger than those of the first type, measuring about 15-20 µm by 8-10 µm. The cell bodies were

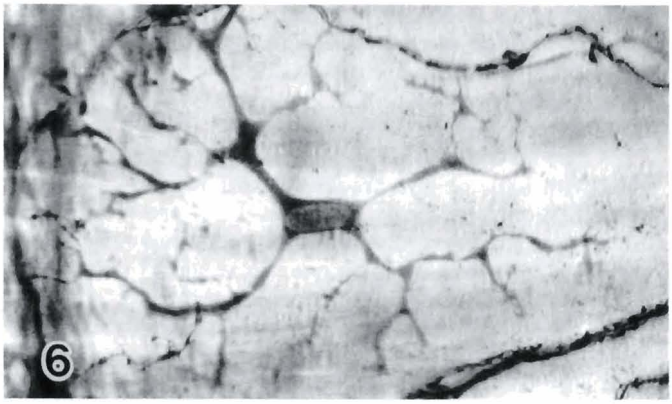

t
s
g
c
s
c
g
t
1

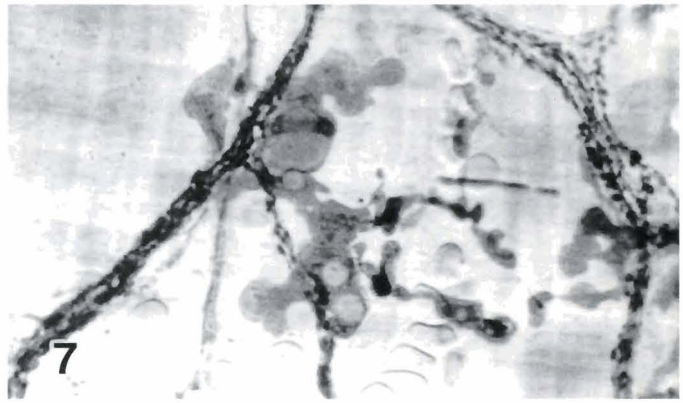

2


3

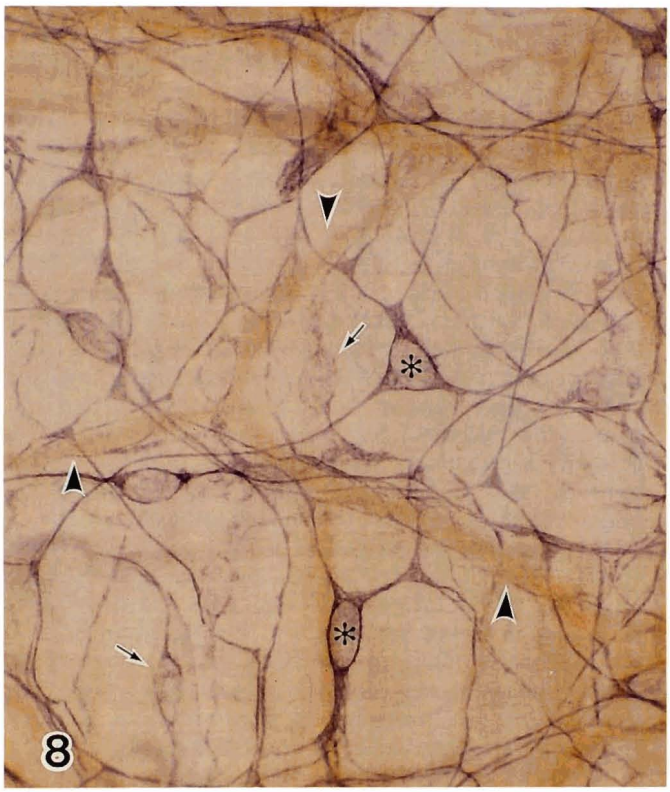

4


5


6


7

located in meshes of the tertiary plexus and did not show close relations with nerve bundles. Three to five primary processes repeated the dichotomy to form secondary, tertiary and further branches. Therefore a single cell of this second type often spanned an area of several hundreds of microns in diameter. These processes connected with the same type of cells to form a distinct cellular network from the nerve plexus. However, in part, the processes appeared to make contacts with nerve bundles of the tertiary plexus. One of the most characteristic features of this cell type was the formation of a triangular knot at every branching point of the processes (Fig. 5). These cells were intensely stained with vimentin immunohistochemistry (Fig. 8). Cells



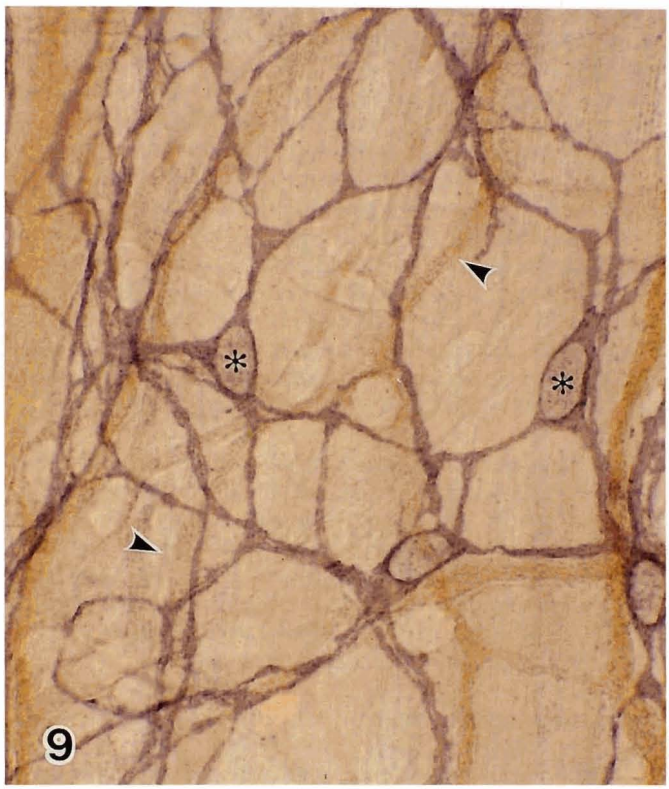


Fig. 8. The second type of myenteric interstitial cells revealed by immunohistochemical staining fro vimentin (*). Note the dichotomous branching pattern of the processes and the triangular knots at the branching points. Nerve bundles of the tertiary plexus are stained brown by the cholinesterase reaction (arrowheads). Independence of the cellular reticulum from the plexus is clear. Weakly-stained cells correspond to the first type of myenteric interstitial cells (arrows). From Komuro and Zhou, 1996. x 650

Fig. 9. Almost exactly the same shape of cells (*) as those in Fig. 8, demonstrated by immunohistochemical staining for c-kit receptor. The tertiary nerve plexus is stained brown by the cholinesterase reaction (arrowheads). From Komuro and Zhou, 1996. x 650

Fig. 1. Myenteric plexus of the guinea-pig small intestine-stained with ZIO method. The fine network of the tertiary plexus (t) is observed in the interstices of the primary plexus constituted by thick ganglion strands (g) and perpendicular connecting strands (c). Several nerve bundles of the secondary plexus (s), which directly connect the primary strands, are seen. ZIO staining. x 120

Fig. 2. A light micrograph showing interstitial cells (arrowheads) over the tertiary plexus and the cells of lymph vessels (arrows). ZIO staining. x 300

Fig. 3. Schwann cells located in the midst of the nerve bundles of the tertiary plexus stained with S-100 immunohistochemistry. x 700

Fig. 4. The first type of myenteric interstitial cells in the guinea-pig small intestine which is located beside the tertiary nerve. It extends several irregular processes. ZIO staining. x 850

Fig. 5. The second type of myenteric interstitial cell characterized by long cytoplasmic processes which show a dichotomous branching pattern. Note triangular knots at branching points (arrows). The cell body is usually seen apart from the nerve bundles. ZIO staining. x 700

Fig. 6. Stellate cells of the lymph vessel. ZIO staining. x 600

Fig. 7. A probable macrophage displaying a different appearance of inclusions revealed by ZIO staining. x 750

having a shape almost exactly similar to those of vimentin-positive cells were demonstrated by *c-kit* receptor immunostaining (Fig. 9). They also showed a dichotomous branching pattern with a triangular knots, and their cellular network was independent from the myenteric plexus.

Many lymph vessels were distributed in the myenteric region, and clusters of stellate cells were observed on the wall of lymph vessels, as confirmed with Nomarski optics (Figs. 2, 6). They were always superimposed on the lymph vessels and confined to the width of the vessels. Cells of this type were similar in size to the first type of interstitial cells, but they differed from the latter in their correlation to nerve bundles. The cell bodies contained round to elongated nuclei and projected the processes in all directions as if they enveloped the wall of the lymph vessel. The processes occasionally showed a dichotomous branching pattern, but they were easily distinguished from the second type because of their irregular contour and angular course. They made contact with each other but they did not appear to form a complete cellular network. Immunoreactivity for vimentin was not observed in cells of this type.

Cells of another type, probably representing macrophages, were scattered in the interstices of the myenteric plexus (Fig. 7). They were elongated cells with a few, short processes and were characterized by many granular structures of different appearance and size. Some of them were vacuolar and others were very dense. These cells tended to be located in the vicinity of blood vessels. They did not have vimentin immunoreactivity.

Circular muscle layer

The circular muscle coat of the guinea-pig small intestine is subdivided into inner thin and outer thick sublayers by the intercalation of the deep muscular plexus (DMP).

Within the outer circular muscle layer, nerve bundles mainly ran parallel to the muscle cells and formed a fairly well-organized plexus with few interconnecting strands (the superficial plexus: SP). Interstitial cells found within this layer were of only one type, which was always well-stained using the ZIO method (Figs 10-13). They were characterized by spindle-shaped cell bodies with a few primary processes. The nuclei were elongated and usually measured about 15 μm by 7 μm.

Their cytoplasmic processes showed different patterns of ramification depending on their relation to nerve bundles. These patterns were roughly classified into three variations, as follows: Cells of the first pattern were observed in association with long, straight portions of nerve bundles which were widely separated from neighboring bundles. They were bipolar cells with extremely long processes and kept a close relationship with nerves throughout the whole extension (Fig. 10). The processes generally showed sharp, wavy contours and a gradual decrease in the caliber towards the tips, though the terminal portions occasionally formed a complex arborization. Bipolar primary processes rarely gave off large secondary processes, but often made fine lateral twigs which penetrated into the muscle tissue.

Cells of the second pattern were seen in association with two parallel nerve bundles and formed an H shape (Fig. 11). The cell bodies attached to one bundle projected a broad primary process perpendicularly towards the neighboring bundles and then gave off bidirectional secondary processes which accompanied the latter. The secondary processes showed similar features to the primary processes of the first pattern.

Cells of the third pattern were found at or near the

Fig. 10. A fusiform interstitial cell beside a thin nerve bundle of SP within the circular muscle layer. Bipolar slender processes are closely associated with varicose nerve fibers. ZIO staining. x 620

Fig. 11. An H-shaped interstitial cell associated with two parallel nerve bundles of SP. An arrow indicates a connecting portion between the parallel processes (arrowheads). ZIO staining. x 650

Fig. 12. A stellate interstitial cell located at the intersection of SP. ZIO staining. x 620

Fig. 13. A stellate cell detached from the nerve bundles. Note that terminal portions of all processes are nearly parallel with each other, which reflects the axis of the circular muscle fibers. ZIO staining. x 620

Fig. 14. A stellate cell within the circular muscle layer demonstrated by the immunohistochemistry for vimentin. x 700

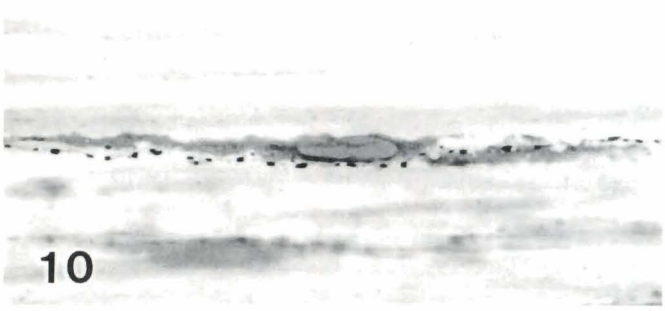
Fig. 15. A drawing of ICC within the rabbit circular muscle stained with methylene blue, adopted from Cajal (1911, Fig. 573). Note the close similarity to Figs. 13 and 14.

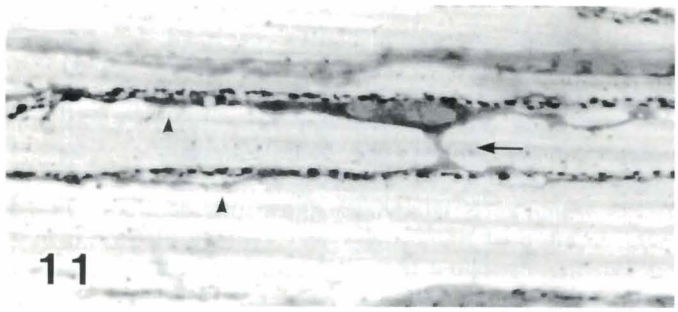
Fig. 16. The first type of interstitial cell in the DMP region. The cell body is located beside the nerves and extends several cytoplasmic processes along the nerve network (arrows). ZIO staining. x 850

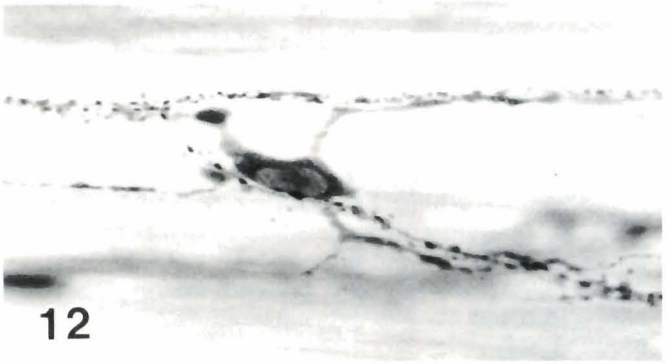
Fig. 17. The second type of interstitial cell in the DMP region. It extends cytoplasmic processes independently from the nerve bundles (arrows). ZIO staining. x 850

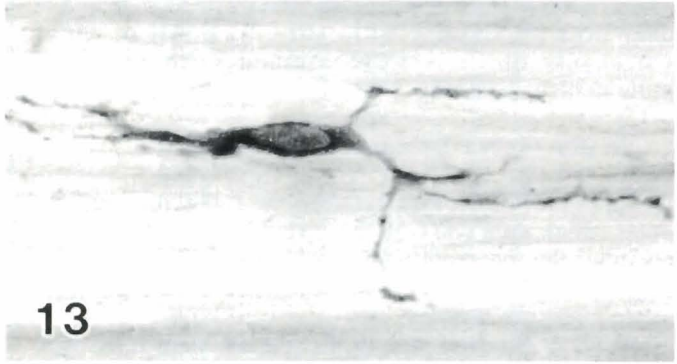
Fig. 18. Immunopositive cells for vimentin antiserum in the DMP region. Note their rounded nuclei and branching processes. x 900

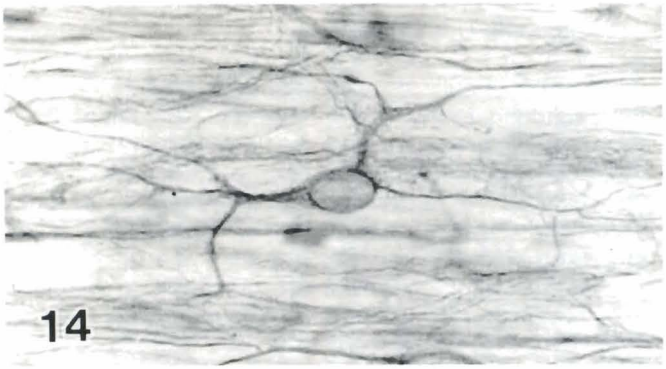
Fig. 19. A glycogen-rich cell stained by Bauer's staining. Arrows indicate its processes. x 800

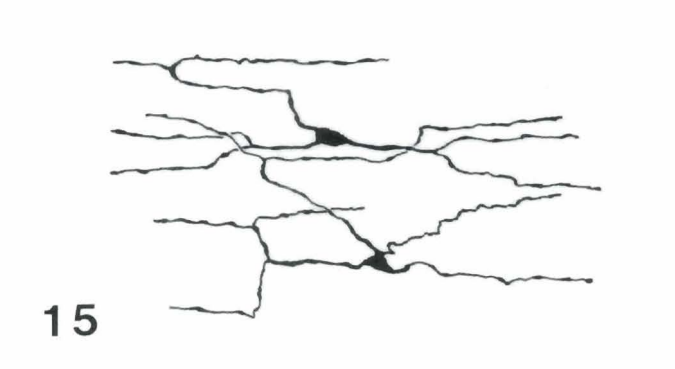

10

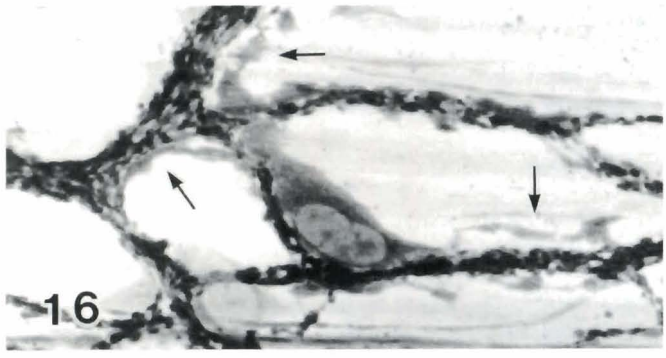

11

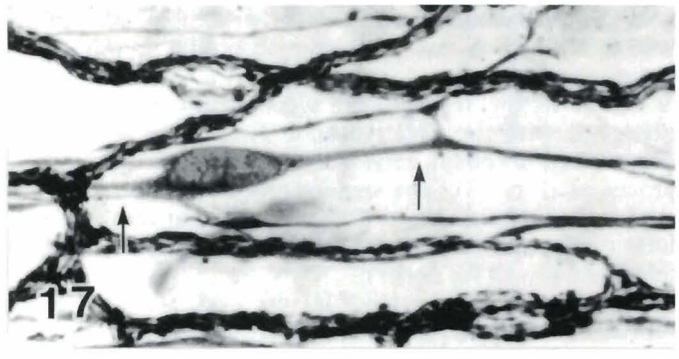

12

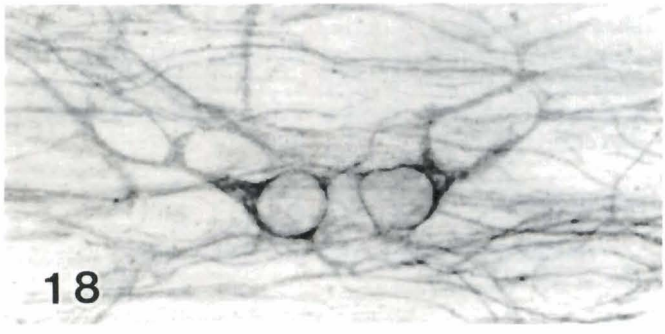

13


14


15


16


17


18


19

intersections of the nerve plexus (Figs. 12, 13). They projected three to five primary processes depending on the number of nerve bundles at these sites. The processes often bridged the gap between the nerve bundles to associate with adjacent ones. Their secondary or tertiary processes occasionally extended along single varicose fibers which originated from these intersections. The whole shape of these cells was clearly observed in specimens in which a part of the nerve plexus had been detached from the cells (Fig. 13). Further, their appearance was almost exactly identical to that of the drawings by Cajal (1911) (Fig. 15). All of these variations of cells were stained with vimentin immunohistochemistry (Fig. 14).

Region of the deep muscular plexus (DMP)

The DMP is located between the inner, thin and the outer, thick sublayers of the circular muscle coat and encircles the intestinal wall. It consists of nerve bundles parallel to the circular muscle fibers and has few interconnecting bundles.

Cells of the first type in this plexus were most well stained with the ZIO method and showed darker gray cytoplasm with paler nuclei (Fig. 16). They were densely distributed throughout the whole network of the nerve bundles. The cell bodies with rich perinuclear cytoplasm were usually elongated and situated beside the nerves. They often appeared to encircle the nerve bundles. The nuclei were also elongated measuring 12-18 μm by 6-10 μm. These cells took a variety of forms depending on the sites of the associated nerve bundles. At straight portions of the nerves, they showed slim spindle shapes with long bipolar processes, while at the intersections the cells projected three to five processes along the courses of the nerves. Their broad processes showed sharp wavy contours and gradually decreased their caliber. In general, the secondary and tertiary processes were not well developed. A few fat droplets could be seen in the cytoplasm.

Cells of the second type were only occasionally observed with the ZIO method. They were spindle-shaped or polygonal cells with three to five primary processes (Fig. 17). The slender processes had smooth contours, and bifurcated and extended for extremely long distances. These processes did not have a close relationship with the nerves.

Cells unstained with ZIO method, which may represent another type of cell, were recognized from their peculiar locations and the shape of the nuclei. Nomarski optics demonstrated a regular distribution of unstained nuclei situated in small loops of nerve bundles, or at/near the intersections. They were distinguished from Schwann cells, which almost always lodge within the midst of the nerve bundles, by their larger size and by the rounded shape of the nuclei.

On the other hand, vimentin immunostaining demonstrated many positive cells with long branching processes (Fig. 18). They often occurred in pairs. They appeared independently from the nerve plexus, though their correlative positions were not clearly identified because of the difficulty of visualizing simultaneously both vimentin-positive cells and the nerve plexus. Their whole cell shape seemed to differ from either the first type or the second type of cells in the DMP. Bauer's staining, however, revealed a regular distribution of glycogen-containing cells which extended long processes (Fig. 19), resembling these vimentin-positive cells.

Along large nerve bundles interconnecting between the myenteric plexus and the superficial plexus or DMP, there were cells resembling the first type of DMP region with respect to staining affinity and close relationship to the nerves. Their features were particularly well observed in the occasional half-stained preparations of the nerves in which they enveloped the nerve bundles with their laminar cytoplasmic process (Fig. 20). A similar appearance of cells was also found in association with blood vessels in the muscle layer (Fig. 21). Here, elongated cells beside the nerve bundles extended a few slender processes along or around the blood vessels.

Longitudinal muscle layer

A small number of interstitial cells were observed in the longitudinal muscle layer with the ZIO method (Fig. 22), though there were few nerve fibers in this layer. Their cell bodies orientated nearly parallel to that of the muscle cells. The nuclei were quite elongated and often measured about 20 μm by 4 μm. These cells were characterized by small perinuclear cytoplasm with several primary processes which occasionally branched off extremely long processes. The slender processes tended to originate directly from the well-demarcated cell bodies and to extend in different directions. The processes showed sharp wavy contours and often formed secondary and tertiary extensions. Fine lateral, twigs were seen throughout the processes. Their immuno-reactivity to vimentin has not been confirmed so far.

Serosa

There were many small stellate cells which were densely and regularly distributed in a two-dimensional plane in the serosa (Fig. 23). They extended many cytoplasmic processes in all directions, which branched off repeatedly. The processes did not extended for a long distance but appeared to make contact with each other. They did not show a definite cell axis, but the longer axis of their cell bodies tended to orientate parallel to that of the longitudinal muscle cells. These cells displayed strong immunoreactivity to vimentin antiserum (Fig. 24).

Discussion

The present observations revealed a wide variety of

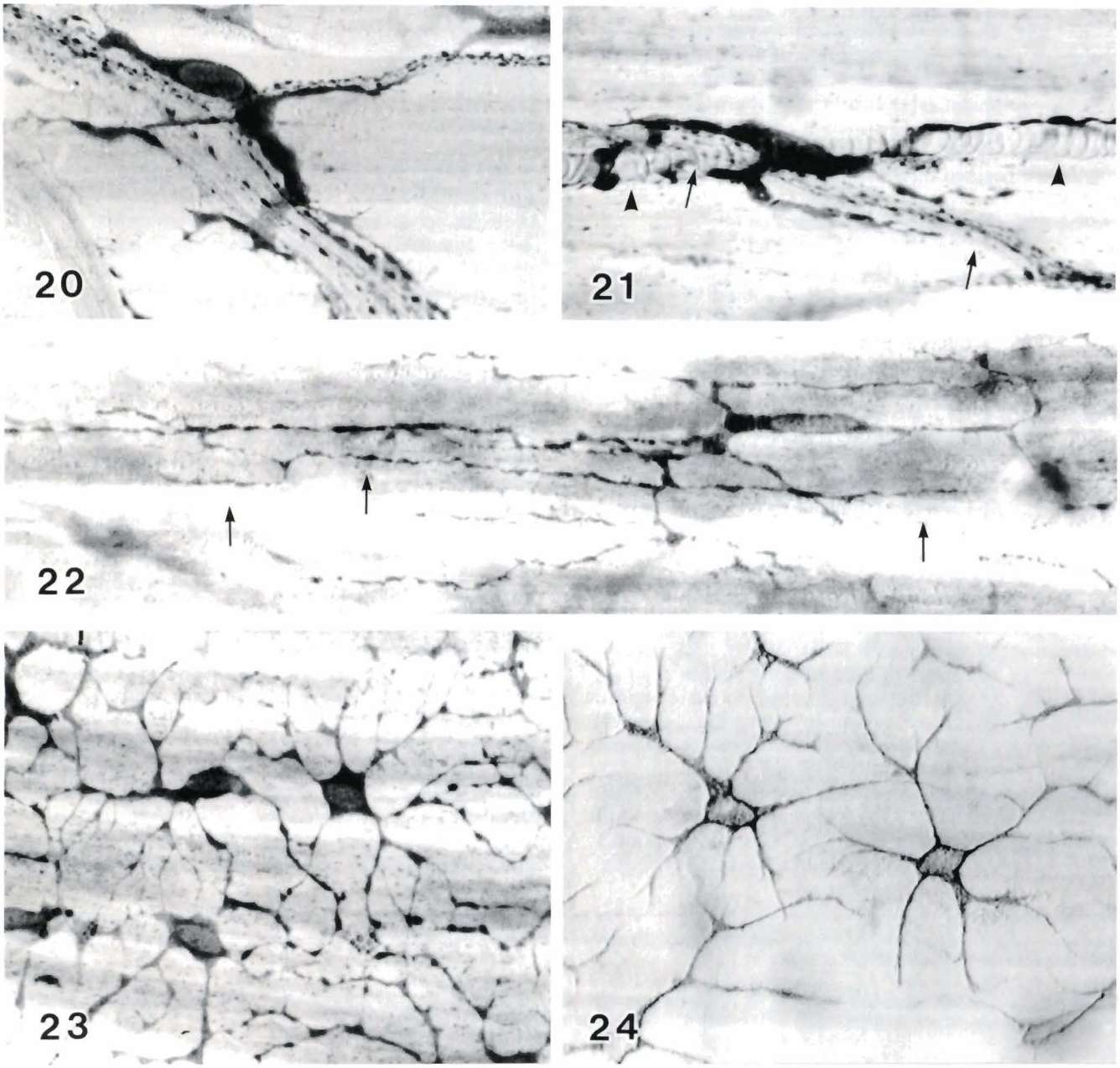


Fig. 20. An interstitial cell associated with an interconnecting nerve bundle between the myenteric plexus and SP. The cell processes envelop a weakly-stained nerve bundle. ZIO staining. x 700

Fig. 21. An interstitial cell associated with a nerve bundle (arrow) and a capillary (arrowhead) in the circular muscle layer. ZIO staining. x 700

Fig. 22. An interstitial cell in the longitudinal muscle layer, which is characterized by poor perinuclear cytoplasm and extremely long branching processes (arrow). Axis of the longitudinal muscle is horizontal. ZIO staining. x 450

Fig. 23. Interstitial cells in the serosa. They extend many branching processes in all directions. ZIO staining. x 600

Fig. 24. Interstitial cells in the serosa demonstrated by immunohistochemistry for vimentin. Their shape and organization is the same as those of Fig. 23. x 650

cells which seem to include the majority of cell types described as ICC in previous reports, by using the ZIO method which stains nerves and a range of interstitial cells. This staining property is common to that of methylene blue or the Golgi method and gives an important clue as to the identity of ICC. The correct location in a particular tissue layer of stained cells and their topographical relationship with the nerves can be identified owing to the distinctive pattern of each nerve plexus. These observations help to classify the interstitial cells on the basis of their morphology and their location.

Their existence as a particular type of cells

Before discussing ICC in detail, the fundamental question about the existence of ICC should be clarified, since it was postulated that the structures which Cajal called interstitial cells in the intestine did not originate from one cell type, but were a chimera composed of the glial cell bodies and the neurites stained simultaneously (Kobayashi et al., 1989).

The present observations clearly demonstrate the presence of several groups of cells which are distinctive in shape and size. For example, the second type in the myenteric region and the stellate cells within the circular muscle layer show their own particular morphology, which is almost identical to the original drawings by Cajal (1911, Fig. 572) and (1911, Fig. 573), respectively. Simultaneous clear visualization of cells of the same shape by vimentin immunohistochemistry (present observation; Komuro and Zhou, 1996) confirms the existence of the specific type of cells, rather than the glia-neurite chimera, because neurites do not contain a substantial amount of vimentin. Therefore, the ICC illustrated by Cajal do not represent merely artificial images, but rather a certain type of cell.

Kobayashi et al. (1986) also postulated that ICC are included in the term enteroglial cells, together with enteric glial cells and Schwann cells, on the basis of S-100 immunostaining of the guinea-pig small intestine. However, it is difficult to categorize these cells into one, since the localization of GFAP, which distinguishes their glial nature (Jessen and Mirsky, 1980), is confined within the myenteric and submucous plexuses and has not been detected on ICC and Schwann cells (Jessen and Mirsky, 1980, 1985; Bjorklund et al., 1984; Nada and Kawana, 1988).

ICC-AP

As mentioned above, the second type of cell in this region demonstrates a characteristic cell shape and forms a cellular network, similar to the ICC described by Cajal (1911, Fig. 572). It is obvious that these cells have been repeatedly observed by many light microscopists. They correspond to the cells of the guinea-pig myenteric plexus stained with methylene blue (Taxi, 1965, Fig. 48), and to the cells of the rabbit stained with silver impregnation (Richardson, 1958, Figs. 1, 3).

Electron microscopic observations revealed that only fibroblast-like cells are the proper interstitial cells (except for the free cells) in this region of the guinea-pig small intestine and that they are well stained with the ZIO method (Zhou and Komuro, 1995; Fig. 25). Their cautious examination suggests that in this region the fibroblast-like cells without a basal lamina can be classified into two subtypes (Komuro and Zhou, 1996). Cells of one type show cytoplasmic features of the typical fibroblast in many respects, but they form small gap junctions with smooth muscle cells (Figs. 26, 27).

On the contrary, cells of another type (Fig. 28) are usually identified by less electron-dense cytoplasm containing many smooth endoplasmic reticulum (sER) components and numerous mitochondria. Fairly well-developed rough endoplasmic reticulum (rER) is also found in the cytoplasm, but its cisterns rarely display dilated forms, unlike those of the former type. The electron-lucent cytoplasmic processes, which are frequently observed between two muscle layers and around the myenteric ganglia (Fig. 29), probably belong

Fig. 25. An electron micrograph showing a fibroblast-like cell (F) stained with the ZIO method between the circular (C) and longitudinal (L) muscle layers in the guinea-pig small intestine. x 9,000

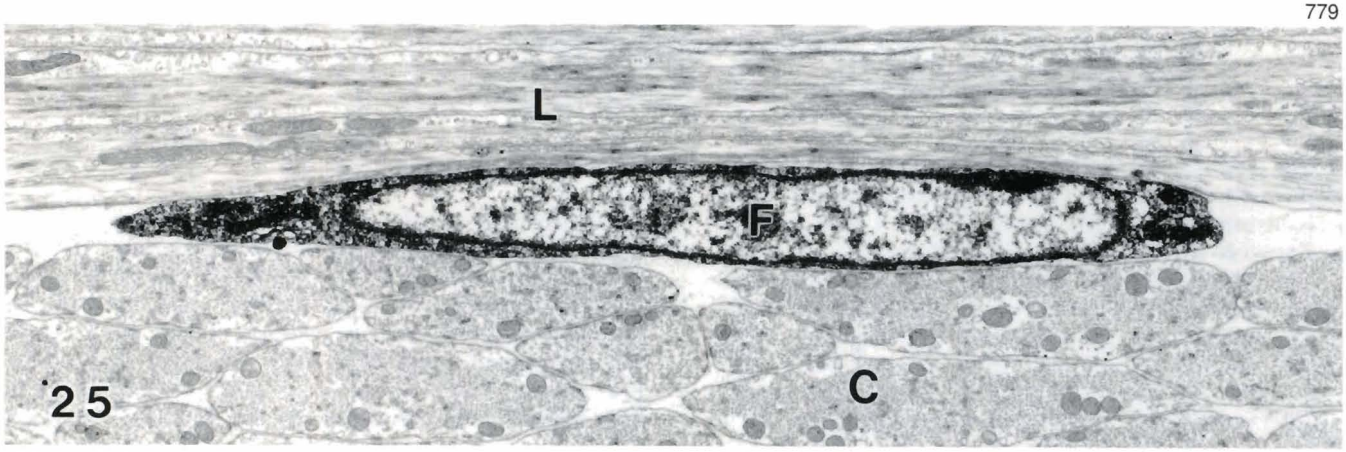
Fig. 26. One type of fibroblast-like cell (F1) in the myenteric region. it makes a small gap junction with circular muscle cells with its small process around the cell body (arrow). Cisterns of rER and several mitochondria are observed in the cytoplasm. x 7,800

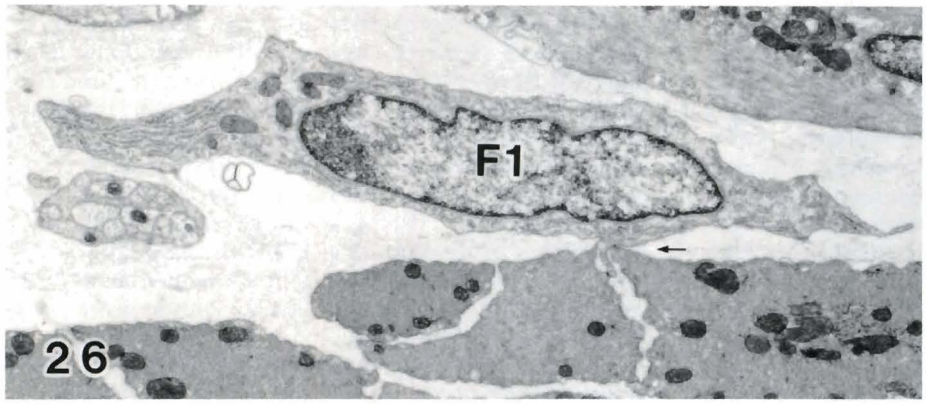
Fig. 27. Higher magnification of the gap junction indicated by the arrow in Fig. 26. x 52,000

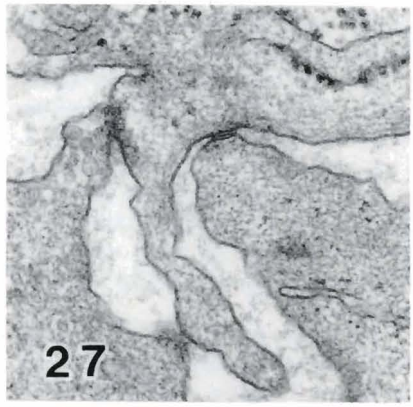
Fig. 28. Another type of fibroblast-like cell (F2) in the myenteric region of the guinea-pig small intestine, which corresponds to ICC-AP. Tubular and vesicular components of sER are observed in the perinuclear cytoplasm. rER is observed in a neighboring cell, which is probably the same type as that in Fig. 26. x 9,800

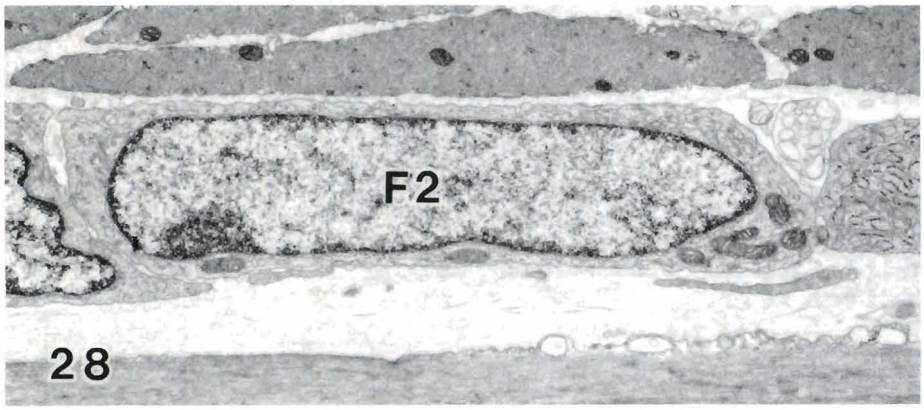
Fig. 29. A gap junction between two processes which are speculated to belong to the same type of cells as those in Fig. 28. They are found in the myenteric region and contain abundant intermediate filaments. They have no basal lamina. x 40,000

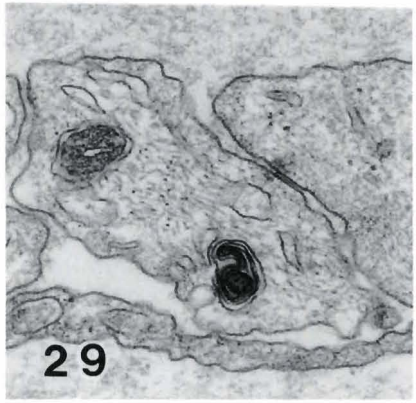
Fig. 30. An electron micrograph showing two subtypes of fibroblast-like cells (F1, F2) between the circular and longitudinal muscle layers in the rat small intestine. «F1» cells are characterized by a more electron-dense cytoplasm and well-developed rER, while «F2» cells have less electron dense cytoplasm and contain more mitochondria and sER components. A small gap junction is observed between «F1» and a tiny process of the muscle cell (arrow). x 10,000. **Insertion:** Higher magnification of the gap junction indicated by the arrow, in a neighboring section to that in Fig. 30 (a, x 31,000), and a large gap junction between two processes speculated to belong to the cell type «F2» (b, x 33,000). Modified from Komuro, 1989.


L
F
C
25


F1
26


27


F2
28


29


b

F1
F2
a
30

to this type of cell, though their direct continuity has not been confirmed so far. These cytoplasmic processes are rich in intermediate filaments, and are connected with one another by fairly large gap junctions.

We speculate that the latter fibroblast-like cells correspond to the ICC in the myenteric region (ICC-AP), described by Cajal (1911), since ICC depicted by the whole mount preparations in the present study show extremely long processes and are connected with one another at their tips. These facts seem to account for the difficulty of observing the continuity between their cell bodies and the slender processes in ultrathin sections. The presence of abundant intermediate filaments in the processes also suggests that they belong to a part of the cells which show strong immunoreactivity to vimentin antiserum. Another cell subtype may correspond to the first type of cell observed by the ZIO method. Although it cannot be ruled out that they represent merely different profiles or functional states of the same type of cell, demonstration of only second type of cell by *c-kit* immunohistochemistry appears to support our speculation.

Cells showing similar ultrastructural features to each of these two subtypes were also distinguished in the myenteric region of the rat small intestine (Komuro, 1989, Fig. 30). The scanning electron micrographs showing well-demarcated cell bodies with slender processes (Komuro, 1989, Fig. 3) probably represent ICC-AP in the rat.

On the other hand, ICC-AP of the mouse intestine (Thuneberg, 1982, 1989) appear to have different fine structural features from those of the guinea-pig. They were described as having an incomplete basal lamina and showing parallel overlapping of the primary processes with nerve bundles of the plexus. Their cytoplasm seems to contain abundant intermediate filaments and numerous mitochondria.

ICC-AP of the human small intestine were reported to show myoid features, including dense bodies, caveolae, basal lamina and well-developed sER, but their gap junctions with one another and with smooth muscle were not observed (Rumessen and Thuneberg, 1991; Rumessen et al., 1993a,b). Berezin et al. (1990) described that ICC-AP of the canine colon had dark condensed cytoplasm and occasional dense bodies. Cells with myoid features were also reported in the human stomach (Faussone-Pellegrini et al., 1989).

The interstitial cells in the guinea-pig small intestine observed by SEM (Baluk and Gabella, 1987; Jessen and Thuneberg, 1991) show intimate association with nerve fascicles of the tertiary plexus, and therefore they probably correspond to the first type of cell in the present observation.

Interstitial cells of the cat intestine depicted by ZIO (Vajda and Feher, 1980) are unique in shape and arrangement and probably correspond to the cell type found on the lymph vessels in the present study. They are most likely to be adventitial fibroblasts.

ICC-CM

Examination of cell shapes found in the outer circular muscle layer offers an important clue for considering cell types and identifying ICC. The cytoplasmic processes of the stellate cells show almost the same branching pattern as those of ICC in the rabbit circular muscle layer (ICC-CM) stained by methylene blue (Cajal, 1911, Fig. 573). It is very likely that these ZIO stained cells correspond to the ICC-CM described by Cajal.

The bipolar cells observed in the present study are similar in shape to the cells of DMP of the mouse small intestine (Rumessen and Thuneberg, 1982) and the cells of the circular muscle layer of the opossum esophagus (Christensen et al., 1987), which are stained by the ZIO method. Differences between ICC-DMP and ICC-CM were stressed in the mouse small intestine (Rumessen and Thuneberg, 1982), in which bipolar cells were regarded as being peculiar to DMP. However, the bipolar cells were frequently observed in close association with varicose nerve fibers in the circular muscle layer of the guinea-pig small intestine.

Bipolar, H-shaped and stellate cells of the circular muscle layer in the guinea-pig intestine are likely to represent structural variations of the same type of cells because of similar staining with the ZIO method, similar range of nuclear size and similar topographical relationship to the nerves. Their different cell shapes appear to be determined by their locations.

Since they are stained with vimentin antiserum, and since only one cell type characterized by fibroblastic ultrastructure is found within the circular muscle layer (Zhou and Komuro, 1992b; Fig. 31), they can be regarded as fibroblast-like cells in nature. Some of these cells are quite similar in shape to the cells of the circular muscle layer of the canine colon, which are visualized by the NADH diaphorase method (Xue et al., 1993). These NADH diaphorase-positive cells were reported to lack a basal lamina.

On the other hand, two types of myoid interstitial cells were distinguished in the human small intestine in different locations within the circular muscle layer (Rumessen et al., 1993a).

ICC-DMP

The first type of cells in the DMP region were always observed beside nerve bundles, and their processes showed a close association with the running course of the nerve bundles. In this respect, they were similar to both ICC-CM, and those located along the connecting nerves between the myenteric and superficial or deep muscular plexus, in the present observation. Cells of similar features were also described in the methylene blue staining preparation by Taxi (1965, Fig. 50). However, these cells do not match the ICC-DMP of the original drawings (Cajal, 1911) in all significant points such as the location of the cell bodies and the

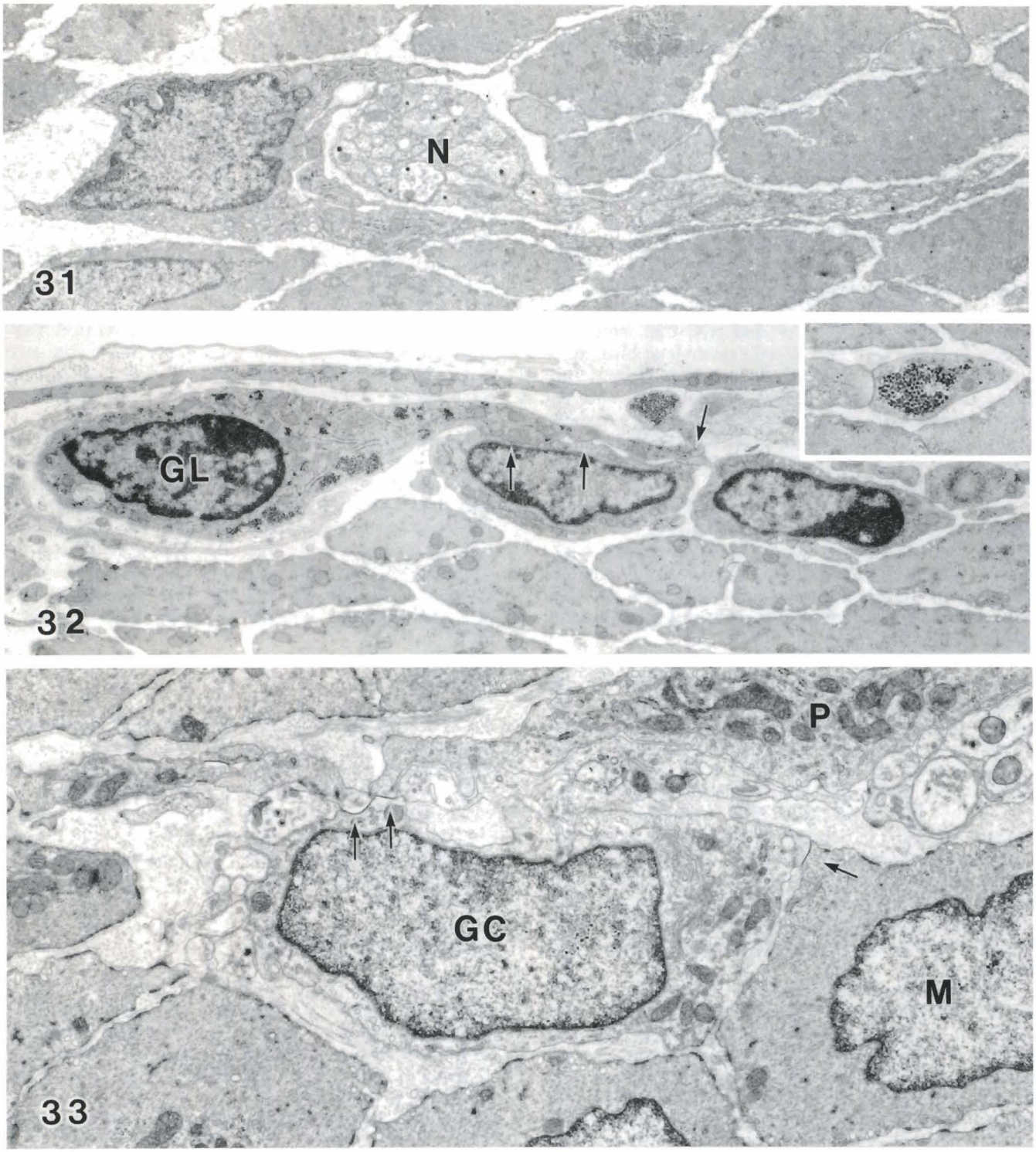


Fig. 31. A fibroblast-like interstitial cell located within the circular muscle layer of the guinea-pig small intestine. There is no basal lamina. N is a nerve bundle. x 9,000

Fig. 32. A glycogen-rich cell (GL) in the guinea-pig DMP region. Its gap junctions with an adjacent cell are indicated by arrows. N is a nerve bundle. x 10,000 I**nsertion:** Higher magnification of the gap junction between the processes of glycogen-rich cells. x 18,000

Fig. 33. A gap junction-rich cell (GC) of DMP region of the rat small intestine. Arrows indicate gap junctions with the processes of the same type of cells (P) and with muscle cell (M). Numerous mitochondria are seen in the processes. Golgi apparatus (G) is located in the paranuclear region. x 12,000. From Komuro and Seki, 1995.

pattern of the branching processes, though they do show some resemblance to them.

ICC-DMP of the guinea-pig small intestine depicted with the Golgi method (Cajal, 1911, Fig. 575) may be different from those of the myenteric region and within the circular muscle layer observed by methylene blue staining. It puzzles us as to how to estimate the exact orientation of the cell bodies of ICC-DMP in the nerve network, though empty spaces in the midst of nerve bundles were indicated for the ICC (Cajal, 1911). ICC-DMP of the guinea-pig were apparently observed under a different staining condition from that used to visualize the whole nerve plexus. These cells may represent a cell type different from ICC-AP and ICC-CM which are stained together with nerves with methylene blue, since different staining effects often result from silver impregnation. This assumption is compatible with the observation that ICC-DMP is never stained by supravital methylene blue in the mouse, and ICC-DMP have not been successfully stained with this method in other species (Thuneberg, 1982).

The present observation indicates the existence of a group of cells which are not stained with the ZIO method, but which are closely associated with nerve bundles. The peculiar location of the cell bodies and size and shape of the nuclei may support the view that they constitute an independent cell type, rather than being simply unstained cells.

We also demonstrated that most of vimentin immunoreactive cells with numerous processes do not match to the figures of the ZIO-stained cells, but appear to correspond to the cells which are stained by Bauer's staining for glycogen. Since the main population of the ZIO-positive cells in DMP (i.e. the first type in the present study) are fibroblast-like cells (Zhou and Komuro, 1995), and since the gap junction-rich cells of the guinea-pig DMP do not to take a stellate form with many processes (Zhou and Komuro, 1992a), glycogen-rich cells (Fig. 32) among three types of interstitial cells in the guinea-pig DMP region (Zhou and Komuro, 1992a,b) are most likely to be ICC-DMP and correspond to the unstained cells with the ZIO method.

If this is the case, the ICC-DMP illustrated by Cajal may represent a unique type of cell, since glycogen-rich cells have not been observed in other species so far. However, it cannot be ruled out that the glycogen-rich cells instead of the gap junction-rich cells in the guinea-pig (Zhou and Komuro, 1992a,b) correspond to a certain population of the myoid cells which form many large gap junctions and are generally regarded as ICC in other species, including mouse (Yamamoto, 1977; Rumessen et al., 1982; Thuneberg, 1982), rat (Komuro and Seki, 1995; Fig. 33) and dog (Duchon et al., 1974; Torihashi et al., 1993). ICC associated with the DMP of human small intestine were reported to resemble smooth muscle cells and to form only occasional small gap junctions (Rumessen et al., 1992).

ICC-SMP

The ICC-SMP of the colon have been considered to correspond to ICC-DMP (Rumessen et al., 1982; Berezin et al., 1988). And the cells similar to those of the mouse DMP (Rumessen et al., 1982) were reported in the dog (Berezin et al., 1988; Torihashi et al., 1994) and in human (Rumessen et al., 1993b), though the gap junctions were not identified in the latter. Their functional implications for pacemaking activity have been well documented (Conklin and Du, 1990; Serio et al., 1990; Liu et al., 1994). However, it is not certain whether they really correspond to the ICC-DMP which were originally described in the guinea-pig small intestine (Cajal, 1911), because the latter cell type itself has not been clearly identified, for the reason just mentioned above.

Interstitial cells associated with guinea-pig SMP are only of one type, i.e., gap junction-rich cells (Ishikawa and Komuro, 1966), whereas three types of interstitial cells have been distinguished in the DMP of the same animal (Zhou and Komuro, 1992a,b). The former does not contain massive glycogen granules, even though the ICC-DMP of the guinea-pig are considered to be the glycogen-rich cells. The cells of SMP are stained with ZIO and vimentin antiserum (Ishikawa and Komuro, 1996).

ICC-LM

The cells of the longitudinal muscle layer show similar staining affinity to the cells of the circular muscle layer in the ZIO method. Although they are different from each other in appearance, it can be assumed that the same type of cells distribute throughout the external muscle coat take different cell shapes adapted to their tissue environment, such as the density of muscle bundles or the pattern of nerves that they associate with.

Interstitial cells of the serosa

Interstitial cells of the serosa in the present study were similar in the pattern of their cytoplasmic processes to those of the mouse stained with methylene blue (Thuneberg, 1982) and with the ZIO method (Rumessen and Thuneberg, 1982), and those of the guinea-pig demonstrated by scanning electron microscopy (Baluk and Gabella, 1987), as well as to cells of the rabbit small intestine stained with silver impregnation (Richardson, 1960). They showed very strong immunoreactivity to vimentin antiserum, and probably represent typical fibroblasts. In this context, it is a puzzling observation that the sub-serosal interstitial cells of the guinea-pig small intestine show strong cyclic GMP immunoreactivity after sodium nitroprusside stimulation (Young et al., 1993).

Functional role of ICC

The discussion in this article is basically confined to the morphological identification of ICC. However, the following brief description will refer to some functional aspects relating to the ICC.

c-kit receptor immunostaining reveals a cellular network which is independent from the myenteric plexus (present observation; Komuro and Zhou, 1996). It consists of characteristically shaped cells which have spindle or triangular cell bodies with slender cytoplasmic processes, showing a dichotomous branching pattern. They closely resemble cells depicted by ZIO method and vimentin immunostaining which we regarded as ICC-AP. Therefore, it can be concluded that ICC-AP correspond to *c-kit* receptor immunoreactive cells.

Since it has been suggested that the *c-kit* receptor is required for the normal development of pacemaker cells (Maeda et al., 1992; Torihashi et al., 1995), and that their defects result in a loss of slow waves (Ward et al., 1994; Huizinga et al., 1995) in the mouse intestine, it is very likely that at least ICC-AP have a pacemaker function. A recent study using *Ws/Ws* mutant rats (Isozaki et al., 1995) also indicated that abnormalities in the ileal movement and pyloric sphincter function are attributable to a deficiency of *c-kit* mRNA-expressing cells.

On the other hand, ICC-DMP probably play a role in the impulse conduction system, as suggested by (Thuneberg, 1982), because they form many gap junctions with one another and with smooth muscle cells in addition to their close contacts with nerve terminals, though no direct physiological evidence has been reported so far.

As to the function of ICC-CM, Thuneberg (1989) reported that their conductive function is questionable, since he had found only intermediate-type junctions with each other and with smooth muscle cells. However, gap junctions have been detected between ICC-CM and smooth muscle cells of the rabbit colon (Komuro, 1982) and of the guinea-pig small intestine (Zhou and Komuro, 1992b). Thus, ICC-CM are quite likely to have a conductive function. Indeed, it was in the gizzard of the love-bird in which the gap junctions between the interstitial cells and smooth muscle cells were found for the first time (Imaizumi and Hama, 1969).

Concluding remarks

The present study clearly demonstrates the real existence of particular types of cells which show exactly the same characteristics as the ICC depicted in the original drawings (Cajal, 1911). It also reveals that ICC described in the myenteric region, DMP and within the circular muscle layer (Cajal, 1911) are ultrastructurally heterogeneous and correspond to the different type of cells in each location.

Regarding the cell nature, Prosser et al. (1989) reported that ICC of the rat small intestine appear to be some type of neuron, because they are stained with neuron specific enolase (NSE) antiserum but are devoid of immunoreactivity for four types of intermediate filaments; GFAP, vimentin, desmin and neurofilament. However, their immunoreactivity seems not to localize to ICC, and no cells immunopositive to NSE were detected in the myenteric and DMP regions of the guinea-pig intestine (Tokui et al., 1992; Zhou and Komuro, 1992a).

ICC-AP, ICC-CM and ICC-LM as well as the interstitial cells of the serosa are probably categorized into fibroblast-like cells. Although ICC-AP with some myoid features were reported in the mouse (Thuneberg, 1982), dog (Berezin et al., 1990; Xue et al., 1993) and human (Faussonne-Pellegrini et al., 1990; Rumessen and Thuneberg, 1991), the present observation clearly demonstrates that ICC-AP and ICC-CM being identical with the original drawings, are fibroblast-like cells. It should be emphasized here that cells with myoid features such as basal lamina and many caveolae have never been identified in the myenteric region of the rat (Komuro, 1989) or the guinea-pig (Zhou and Komuro, 1995) small intestine. A cell type resembling the typical fibroblast with small gap junctions was further observed in the myenteric region of the rabbit colon (Komuro, 1982). Thus, if all these species really have equivalent cell types in corresponding locations, those ICC-AP with a different appearance can be considered as species variations of the fibroblast-like cells rather than those of smooth muscle cells, since it has been well documented that a family of fibroblasts shows a wide diversity of cytological features and that differences among these cells are quantitative rather than qualitative (Komuro, 1990).

Those cells probably represent a subtype of fibroblasts which are specialized for intercellular communication (and/or pacemaker function) in the smooth muscle tissue, and display a different degree of fibroblastic-myoid features depending on their microenvironment, as determined by tissue layer, or level of the alimentary tract, or species.

On the other hand, since some myoid features were shown in every putative ICC-DMP from different species, including mouse (Yamamoto, 1977; Rumessen et al., 1982; Thuneberg, 1982), rat (Komuro and Seki, 1995), guinea-pig (Zhou and Komuro, 1992a,b), dog (Duchon et al., 1974; Torihashi et al., 1993) and human (Rumessen et al., 1992), it is very likely that ICC-DMP belong to a special type of smooth muscle cells. Furthermore, ICC-DMP show more myoid features, including dense bodies and better developed basal lamina, as well as caveolae and subsurface cisterns in species with a larger body size (dog, human) than those with smaller body size (mouse, rat, guinea-pig).

Novotny and Gnoth (1991) demonstrated considerable differences in size and morphology of fibroblasts in the human digital dermis and subcutis by using silver impregnation. Further, Wake and Sato (1993) reported that the perisinusoidal stellate cells (fat-

storing cells) of the porcine liver display marked morphological heterogeneity in their location in the hepatic lobule, using the Golgi method. These observations may explain why the ZIO method, which is generally regarded as a specific staining method for nervous elements, revealed a wide variety of interstitial cells in different tissue layers including typical serosal fibroblasts in the present study.

It is an important issue for future studies to characterize the functional significance of ICC in each location.

Acknowledgements. Authors thank Dr. P. Baluk, Cardiovascular research Institute, University of California, San Francisco, USA, for reading of the manuscript.

References

Anderson C.R. and Edwards S.L. (1993). Subunit b of cholera toxin labels interstitial cells of Cajal in the gut of rat and mouse. Histochemistry 100, 457-464.

Baluk P. and Gabella G. (1987). Scanning electron microscopy of the muscle coat of the guinea-pig small intestine. Cell Tissue Res. 250, 551-561.

Berezin I., Huizinga J.D. and Daniel E.E. (1988). Interstitial cells of Cajal in the canine colon: a special communication network at the inner border of the circular muscle. J. Comp. Neurol. 273, 42-51.

Berezin I., Huizinga J.D. and Daniel E.E. (1990). Structural characterization of interstitial cells of Cajal in myenteric plexus and muscle layers of canine colon. Can. J. Physiol. Pharmacol. 68, 1419-1431.

Bjorklund H., Dahl D. and Seiger A. (1984). Neurofilament and glial fibrillary acid protein-related immunoreactivity in rodent enteric nervous system. Neuroscience 12, 277-287.

Boeke J. (1949). The sympathetic end formation, its synaptology, the interstitial cells, the periterminal network, and its bearing on the neurone theory. Discussion and critique. Acta Anat. 8, 18-61.

Cajal S.R. (1893). Sur les ganglions et plexus nerveux de l'intestin. Compt. Rend. Soc. Biol. Paris 45, 217-223.

Cajal S.R. (1911). Histologie du systeme nerveux de l'homme et des vertebres. Tome 2. Maloine. Paris.

Christensen J. (1992). A commentary on the morphological identification of interstitial cells of Cajal in the gut. J. Auton. Nerv. Syst. 37, 75-88.

Christensen J., Rick G.A. and Soll D.J. (1987). Intramural nerves and interstitial cells revealed by the Champy-Maillet stain in the opossum esophagus. J. Auto. Nerv. Syst. 19, 137-151.

Conklin J.L. and Du C. (1990). Pathways of slow-wave propagation in proximal colon of cats. Am. J. Physiol. 258 (Gastrointest. Liver Physiol. 21). G894-G903.

Du C. and Conklin J.L. (1989). Origin of slow waves in the isolated proximal colon of the cat. J. Auton. Nerv. Syst. 28, 167-178.

Duchon G., Henderson R. and Daniel E.E. (1974). Circular muscle layers in the small intestine. In: Proc. 4th Int. Symp. Gastrointest. Daniel E.E. (eds). Motil. Banff, Alberta, Canada. Mitchell Press. Vancouver. pp 635-646.

Faussone-Pellegrini M.S. (1984). Morphogenesis of the special circular muscle layer and of the interstitial cells of Cajal related to the plexus muscularis profundus of mouse intestinal muscle coat. An EM study. Anat. Embryol. 169, 151-158.

Faussone-Pellegrini M.S. (1985). Cytodifferentiation of the interstitial cells of Cajal related to the myenteric plexus of mouse intestinal muscle coat. An EM study from fetal to adult life. Anat. Embryol. 171, 163-169.

Faussone-Pellegrini M.S. (1987). Cytodifferentiation of the interstitial cells of Cajal of mouse colonic circular muscle layer: An EM study from fetal to adult life. Acta Anat. 128, 98-109.

Faussone-Pellegrini M.S., Pantalone D. and Cortesini C. (1989). An ultrastructural study of the interstitial cells of Cajal of the human stomach. J. Submicrosc. Cytol. Pathol. 21, 439-460.

Faussone-Pellegrini M.S., Cortesini C. and Panetalone D. (1990). Neuromuscular structures specific to the submucosal border of the human colonic circular muscle layer. Can. J. Physiol. Pharmacol. 68, 1437-1446.

Hara Y., Kubota M. and Surszerszewski J.H. (1986). Electrophysiology of smooth muscle of the small intestine of some mammals. J. Physiol. 372, 501-520.

Huizinga J.D., Shin A. and Chow E. (1988). Electrical coupling and pacemaker activity in colonic smooth muscle. Am. J. Physiol. 255 (Cell Physiol. 24), C653-C660.

Huizinga J.D., Thuneberg L., Kluppel M., Malysz J., Mikkelsen H.B. and Berstein A. (1995). W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature 373, 341-349.

Imaizumi M. and Hama K. (1969). An electron microscopic study on the interstitial cells of the gizzard in the love-bird (*Uroloncha domestica*). Z. Zellforsch. 97, 351-357.

Ishikawa K. and Komuro T. (1996). Characterization of the interstitial cells associated with the submuscular plexus of the guinea-pig colon. Anat. Embryol. (in press).

Isozaki K., Hirota S., Nakama A., Miyagawa J., Shinomura Y., Xu Z.D., Nomura S. and Kitamura Y. (1995). Distributed intestinal movement, bile reflux to the stomach, and deficiency of c-kit-expressing cells in Ws/Ws mutant rats. Gastroenterology 109, 456-463.

Jessen K.R. and Mirsky R. (1980). Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature 286, 736-737.

Jessen K.R. and Mirsky R. (1985). Glial fibrillary acidic polypeptides in peripheral glia. Molecular weight, heterogeneity and distribution. J. Neuroimmunol. 8, 377-393.

Jessen H. and Thuneberg L. (1991). Interstitial cells of Cajal and Auerbach's plexus. A scanning electron microscopical study of guinea-pig small intestine. J. Submicrosc. Cytol. Pathol. 23, 195-212.

Karnovsky M.J. and Roots L. (1964). A direct-coloring thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219-221.

Keith A. (1914/1915). An account of six specimens fo the great bowel removed by operation: with some observations on the motor mechanism of the colon. Br. J. Surg. 2, 576-599.

Keith A. (1915). A new theory of the causation of enterostasis. Lancet 2, 371-375.

Keith A. (1916). Proceedings of the Anatomical Society of Great Britain and Ireland. J. Anat. Physiol. 50, 1-3.

Kobayashi S., Suzuki M., Endo T., Tsuji S. and Daniel E.E. (1986). Framework of the enteric nerve plexuses: an immunocytochemical study in the guinea pig jejunum using and antiserum to S-100 protein. Arch. Histol. Jpn. 49, 159-188.

Kobayashi S., Furness J.B., Smith T.K. and Pompolo S. (1989). Histological identification of the interstitial cells of Cajal in the guinea-pig small intestine. Arch. Histol. Cytol. 52, 267-286.

Komuro T. (1982). The interstitial cells in the colon of the rabbit: scanning and transmission electron microscopy. Cell Tissue Res. 222, 41-51.

Komuro T. (1987). Three-dimensional structure of the intestinal wall. Thera Res. 7, 107-115.

Komuro T. (1989). Three-dimensional observation of the fibroblast-like cells associated with the rat myenteric plexus, with special reference to the interstitial cells of Cajal. Cell Tissue Res. 255, 343-351.

Komuro T. (1990). Re-evaluation of fibroblasts-like and fibroblast cells. Anat. Embryol. 182, 103-112.

Komuro T. and Seki K. (1995). Fine structural study of interstitial cells associated with the deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells. Cell Tissue Res. 282, 129-134.

Komuro T. and Zhou D.S. (1996). Anti-c-kit protein immunoreactive cells corresponding to the interstitial cells of Cajal in the guinea-pig small intestine. J. Auto. Nerv. Syst. (in press).

Komuro T., Zhou D.S. and Tokui K. (1994). Identification of the interstitial cells of Cajal. XIV Federative International Congress of Anatomy. Lisbon, p 169. (Abstracts).

Liu L.W.C., Thuneberg L., Daniel E.E. and Huizinga J.D. (1993). Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. Am. J. Physiol. 264 (Gastrointest. Liver Physiol. 27), G64-G73.

Liu L.W.C., Thuneberg L. and Huizinga J.D. (1994). Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. Am. J. Physiol. 266 (Gastrointest. Liver Physiol. 29), G4854-G496.

Maeda H., Yamagata A., Nishikawa S., Yoshinaga K., Kobayashi S., Nishi K. and Nishikawa S. (1992). Requirement of c-kit for development of intestinal pacemaker system. Development 116, 369-375.

Meyling H.A. (1953). Structure and significance of the peripheral extension of the autonomic nervous system. J. Comp. Neurol. 99, 495-543.

Nada O. and Kawana T. (1988). Immunohistochemical identification of supportive cell types in the enteric nervous system of the rat colon and rectum. Cell Tissue Res. 251, 523-529.

Novotny G.E.K. and Gnoth C. (1991). Variability of fibroblast morphology *in vivo*: a silver impregnation study on human digital dermis and subcutis. J. Anat. 177, 195-207.

Prosser C.L., Holzwarth M.A. and Barr L. (1989). Immunocytochemistry of the interstitial cells of Cajal in the rat intestine. J. Auton. Nerv. Syst. 27, 17-25.

Richardson K.C. (1958). Electron microscopic observations on Auerbach's plexus in the rabbit, with special reference to the problem of smooth muscle innervation. Am. J. Anat. 103, 99-135.

Richardson K.C. (1960). Studies on the structure of autonomic nerves in the small intestine, correlating the silver-impregnated image in light microscopy with the permanganate-fixed ultrastructure in electron microscopy. J. Anat. 94, 457-472.

Rumessen J.J. and Thuneberg L. (1982). Plexus muscularis profunds and associated interstitial cells. I. Light microscopical studies of mouse small intestine. Anat. Rec. 203, 115-127.

Rumessen J.J. and Thuneberg L. (1991). Interstitial cells of Cajal in human small itnestine. Ultrastructural identification and organization between the main smooth muscle layers. Gastroenterology 100, 1417-1431.

Rumessen J.J., Thuneberg L. and Mikkelsen H.B. (1982). Plexus muscularis profunds and associated interstitial cells. II. Ultrastructural studies of mouse small intestine. Anat. Rec. 203, 129-146.

Rumessen J.J., Mikkelsen H.B. and Thuneberg L. (1992). Ultrastructure of interstitial cells of Cajal associated with deep muscular plexus of human small intestine. Gastroenterology 102, 56-88.

Rumessen J.J., Mikkelsen H.B., Qvortrup K. and Thuneberg L. (1993a). Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. Gastroenterology 104, 343-350.

Rumessen J.J., Peters S. and Thuneberg L. (1993b). Light and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. Lab. Invest. 68, 481-495.

Serio R., Huizinga J.D., Barajas-Lopez C. and Daniel E.E. (1990). Interstitial cells of Cajal and slow wave generation in canine colonic circular muscle. J. Auton. Nerv. Syst. 30, S141-S144.

Shuttleworth C.W., Xue C., Ward S.M., De Vente J. and Sanders K.M. (1993). Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. Neuroscience 56, 513-522.

Suzuki M., Prosser C.L. and Dahms V. (1986). Boundary cells between longitudinal and circular layers: essential for electrical slow waves in cat intestine. Am. J. Physiol. 250, G287-G294.

Taxi J. (1965). Contribution a l'etude des connexions des neurones moteurs du systeme nervoux autonome. Ann. Sci. Nat. Zool. Tome VII, pp 413-474.

Thuneberg L. (1982). Interstitial cells of Cajal: intestinal pacemaker cells? Adv. Anat. Embryol. Cell. Biol. 71, 1-30.

Thuneberg L. (1989). Interstitial cells of Cajal. In: Handbook of physiology. The gastrointestinal system. Vol. 1. Woods J.D. (ed.) American Physiology Society. Bethesda, Maryland. pp 349-386.

Thuneberg L., Rumessen J.J. Mikkelsen H.B., Peters S. and Jessen H. (1995). Structural aspects of interstitial cells of Cajal as intestinal pacemaker cells. In: Pacemaker activity and intercellular communication. Huizinga J.D. (ed.). CRC Press. Boca Raton. pp 193-222.

Tokui K., Zhou D.S. and Komuro T. (1992). Immunohistochemical study of the interstitial cells of Cajal. J. Smooth Muscle Res. 28, 380-382.

Torihashi S., Kobayashi S., Gerthoffer W.T. and Sanders K.M. (1993). interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. Am. J. Physiol. 265, G638-G645.

Torihashi S., Gerthoffer W.T., Kobayashi S. and Sanders K.M. (1994). Identification and classification of interstitial cells in the canine proximal colon by ultrastructure and immunohistochemistry. Histochemistry 101, 169-183.

Torihashi S., Ward S.M., Nishikawa S., Nish K., Kobayashi S. and Sanders K.M. (1995). *c-kit* dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res. 280, 97-111.

Vajda J. and Feher E. (1980). Distribution and fine structure of the interstitial cells of Cajal. Acta Morphol. Acad. Sci. Hung. 28, 251-258.

Wake K. and Sato T. (1993). Intralobular heterogeneity of perisinusoidal stellate cells in porcine liver. Cell Tissue Res. 273, 227-237.

Ward S.M., Burns A.J., Torihashi S. and Sanders K.M. (1994). Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. J. Physiol. (London) 480, 91-97.

Xue C., Ward S.M., Shuttleworth W. and Sanders K.M. (1993). Identification of interstitial cells in canine proximal colon suing NADH diaphorase histochemistry. Histochemistry 99, 373-384.

Xue C., Pollock J., Schmidt H.H.H.W., Ward S.M. and Sanders K.M. (1994). Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. J. Auto. Nerv. Syst. 49, 1-14.

Yamamoto M. (1977). Electron microscopic studies on the innervation of the smooth muscle and the interstitial cell of Cajal in the small intestine of the mouse and bat. Arch. Histol. Jpn. 40, 171-201.

Young H.M., McConalogue K., Furness J.B. and De Vente J. (1993). Nitric oxide targets in the guinea pig intestine identified by induction of cyclic GMP immunoreactivity. Neuroscience 56, 513-522.

Zhou D.S. and Komuro T. (1992a). Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res. 268, 205-216.

Zhou D.S. and Komuro T. (1992b). The cellular network of interstitial cells associated with the deep muscular plexus of the guinea pig small intestine. Anat. Embryol. 186, 519-527.

Zhou D.S. and Komuro T. (1995). Ultrastructure of the zinc iodide-osmic acid stained cells in guinea pig small intestine. J. Anat. 187, 481-485.